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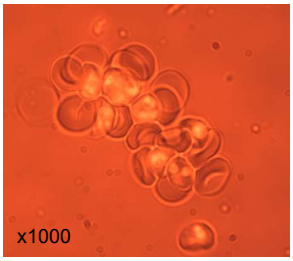
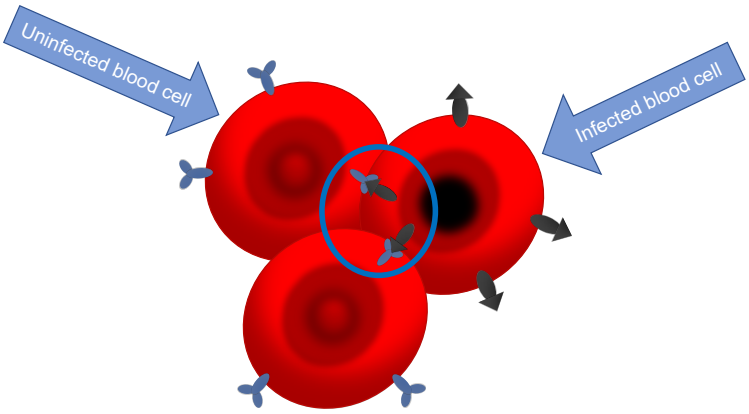
Investigation of host red blood cell receptors essential for rosetting in severe *Plasmodium falciparum* malaria

Dr Fiona Roisin McQuaid





Fiona McQuaid 3 Minute thesis 2019

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I. Abstract

Plasmodium falciparum malaria kills almost half a million people every year, many of whom are children living in Africa. Rosetting is a pathological phenomenon which is associated with all types of severe malaria and occurs when two or more uninfected erythrocytes adhere to an erythrocyte infected with the mature form of the *P. falciparum* parasite. It is thought that these rosettes may cause obstruction of the microvasculature leading to the serious complications seen in severe malaria. Understanding the molecular mechanisms of rosetting could therefore lead to the development of new adjuvant therapies for severe disease. The overall aims of this work were to reassess the evidence for previously described host erythrocyte rosetting receptors, explore new methods of investigating rosetting mechanisms, including generating knockdown/out erythrocytes from CD34+ haematopoietic stem cells and immortalised erythroid precursors, and to identify novel rosetting receptors.

This thesis begins by reassessing the evidence that the glycosaminoglycans, heparan sulfate (HS) and chondroitin sulfate (CS) are involved in rosetting. Contrary to previously published work, results from experiments using carefully validated enzymes to cleave HS or CS did not support the hypothesis that HS or CS are important host receptors for rosetting across the six *P. falciparum* strains tested. In addition, I found no evidence to suggest that HS or CS are actually present on mature erythrocytes, though HS was detected on early bone marrow-derived erythrocyte precursors.

Secondly, I investigated the use of induced pluripotent stem cells (iPSC) and cells cultured from adult bone marrow CD34+ stem cells (cRBC) as a tool to produce knockdown erythrocytes using RNA inference techniques. The cRBC appeared, both morphologically and by receptor profiling with flow cytometry, to be a good approximation of reticulocytes. However, unexpectedly, the cRBC derived erythrocytes were only able to form

rosettes with two of the four parasite lines tested. Further study into the subtle differences in receptor expression levels between cRBC and peripheral erythrocytes suggested that Band 3 could be a potential novel rosetting receptor. This hypothesis was supported by the results of rosette disruption experiments which showed that antibodies to the Wright^{b} antigen, carried on Band 3, were capable of significantly disrupting rosettes of mature erythrocytes across all parasites strains tested.

Finally, I used a new, immortalised erythroid precursor line, the “EJ” cells, developed by the Duraisingh laboratory at Harvard university, to further investigate the role of Band 3 and the Wright^{b} antigen in rosetting. Band 3, glycophorin A (GYPA) and CR1 knockout EJ cells, created using CRISPR/Cas9 technology, were tested for rosetting ability with six parasite lines. As the Wright^{b} antigen requires both Band 3 and GYPA to properly form, both these knockout EJ cells also lacked Wright^{b}. Compared to wildtype EJ cells, GYPA and Band 3 knockout EJ cells had reduced rosetting. However, the rosetting rates were similar between the two knockout lines, suggesting that neither the presence of GYPA or Band 3 alone can rescue the poor rosetting phenotype in the absence of Wright^{b}.

In summary, I have found that while there is little evidence to support the involvement of HS or CS in rosetting, the Wright^{b} antigen carried on Band 3 may be an important, strain-transcending rosetting receptor and could represent a useful therapeutic target to reduce rosetting. In addition, I have developed new techniques for investigating rosetting receptors using a novel erythroid precursor line. The EJ cells also have great potential for the development of a rosetting screen to identify other candidates to help reduce the mortality and morbidity of severe malaria in the future.

II. Lay abstract

There are over 200 million cases of malaria every year and almost half a million deaths, most of which are in children living in Africa. While the majority of children who get malaria suffer a ‘flu-like’ illness from which they recover well, around one in ten will develop severe, life-threatening symptoms. It is therefore important to discover what is different about these severe cases compared to the uncomplicated ones. A particular feature which is seen more commonly in severe malaria is that when a red blood cell is infected with the malaria parasite, it develops sticky projections on the outside of the cell, which allow it to latch onto other red blood cells which have not yet been infected. This causes clumps of infected and uninfected cells to form and these clumps are called rosettes. Rosettes are thought to cause obstructions in the tiny blood vessels throughout the body, leading to the life-threatening symptoms of severe malaria. At present, none of the current medications used to treat malaria are able to break apart these rosettes and there is an urgent need to better understand how rosettes develop in order to design new treatments specifically for severe malaria.

For my thesis, I was particularly interested in the tiny structures on the *uninfected* red blood cells that the parasite infected cells use to latch on. I began by studying two structures called heparan sulfate and chondroitin sulfate, which other researchers had suggested might be important. I found that removing these structures, with chemicals known as enzymes, made no difference to how well the red cells formed rosettes. I also found that the red blood cells which flow around our body probably do not have any heparan sulfate or chondroitin sulfate on their cell surface. This suggests that neither of these structures are important for rosetting.

I then decided to develop another method for investigating which structures are important for rosetting, by generating ‘designer’ red blood cells, allowing me to choose which structures were present on the cell surface. To begin

with, I used cells from adults who had donated their bone marrow. Unexpectedly, I found that red blood cells grown from these cells did not form rosettes very well. By comparing them to ‘normal’ red blood cells, I realised that they had less of a specific structure called Band 3, one part of which is called Wright^{b}. If I blocked Wright^{b} on ‘normal’ red blood cells, I discovered I was able to break apart rosettes in lots of different types of malaria.

I then worked with scientists from Harvard University to grow immature forms of red cells which had the instructions for making Wright^{b} cut out from their genetic code, using a new technique known as CRISPR/Cas9 genome editing. This means that these cells do not have any Wright^{b} on their cell surface. When I tested the Wright^{b}-free cells with different malaria parasites I found they were not able to form rosettes very well, which suggests that Wright^{b} is indeed key for rosetting in malaria. This is fantastic news, as it means that Wright^{b} could be a useful target for medications designed to reduce rosetting in severe malaria. The next phase of my research is to further investigate how the malaria parasite uses Wright^{b} to form rosettes, and whether we can design a therapy to reduce the burden of severe malaria in the future.

III. Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own.

Fiona Roisin McQuaid

September 2019

IV. Acknowledgments

So many people have contributed towards my development from lab-naïve medic to semi-competent scientist and I am extremely grateful to them all. I am also indebted to the Wellcome Trust for funding this project.

First and foremost, my supervisor Professor Alex Rowe, whose patient support, kindness, encouragement and phenomenal intelligence knows no limits. She promised me it would work out in the end, and despite my doubts, it has. She is a fantastic role model for all scientists, but particularly women and I am glad to be part of her team.

Speaking of which, the Rowe lab, past and present has been a great source of comradeship and guidance (and commiseration when the cultures got infected): Martha Cooper and Ahmed Raza, who guided my first, hesitant baby-steps in the lab, Yonxy Azasi, Kathryn Milne, Robert Donat, Megan Cole, my fellow medic Flo Mclean and Eden Taylor, the fantastic honours student who helped me with the early Wright^{b} experiments. Finally, my wonderful peer-mentor, Livvy Swann, whose constant enthusiasm has encouraged me all the way and I have hugely benefitted from her pioneering the way as an ECAT paediatrician/scientist/mother.

My second supervisor, Professor Lesley Forrester, has been a great source of advice regarding the haematological aspects of my thesis and I am grateful to those in her lab who assisted with the iPSC work and gave advice including Richard Axton, Helen Taylor and Martha Lopez Yrigoyen. Also, to Dr Jo Mountford for providing the cRBC culturing protocols.

The ECAT team of directors, particularly Dr Mandy Drake, have given me a fantastic opportunity and Jo Ness has gone above and beyond to smooth the way and provide reassuring guidance at many points.

The team at Harvard who welcomed me, taught me and provided the EJ cells; Professor Manoj Duraisingh, Erik Scully, Estela Shabani, and particularly Becca Lee who patiently supervised me during my time in the Duraisingh lab.

Within King's Buildings, Dr Martin Waterfall has provided invaluable help with flow cytometry and Dr Dave Kelly with microscopy. Those in my room (4.62); Balazs Szoor, Mathieu Cayla and Eleanor Silvester have endured multiple basic scientific questions and provided fantastic companionship over the past 3-4 years for which I am very grateful.

On a personal level I believe that God has brought me here for a reason and carried me through the challenging times, so "to him who is able to do immeasurably more than we could ask or imagine... to him be the glory" (Ephesians 3v20-21). I am also incredibly privileged to have the most wonderful parents, Gillian and Ronald McQuaid, who have supported me the longest and continue to do so in many ways. They taught me so much of what I know and have helped me get where I am today.

And finally, Tim, my wonderful husband, and Esther, the best and most important result of the past 3 years. You bring me joy and balance, thank you.

V. List of Abbreviations

^{o}C	Degrees centigrade
BEL-A	Bristol Erythroid Line Adult
BM	Bone marrow
BSA	Bovine serum albumin
dH20	Distilled water
CAS	CRISPR associated
CIDR	Cysteine rich interdomain regions
CM	Cerebral malaria
cRBC	Cultured red blood cells originating from CD34+ haematopoietic stem cells
CRISPR	Clustered regularly interspaced short palindromic repeats
CS	Chondroitin sulfate
DAPI	4',6-Diamidino-2-phenylindole
DBL	Duffy-like binding domain
DC	Domain cassette
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
EJ	Immortalised erythroid cells, developed by and named after Dr Erik James Scully
EPO	Erythropoietin
Fab	Antigen-binding antibody fragment
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum
GAG	Glycosaminoglycan
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GYPA	Glycophorin A
GYPB	Glycophorin B
GYPC	Glycophorin C
Hb	Haemoglobin

HBEC	Human brain endothelial binding cells
HiDEP	Human iPSC cell-derived erythroid progenitor cells
HDR	Homology directed repair
HS	Heparan sulfate
HSC	Haematopoietic stem cell
HUDEP	Human umbilical cord blood -derived erythroid progenitor cells
IFA	Immunofluorescence assay
iPSC	Induced pluripotent stem cell
KO	Knock-out
MACS	Magnetic Activated Cell Sorting
MOI	Multiplicity of infection; the number of transducing viral units per cell
mRNA	messenger RNA
NHEJ	Non-homologous end joining
NTS	N-terminal segment
PAGE	Polyacrylamide gel electrophoresis
PAM	Protospacer adjacent motif
PAR	Palo Alto, rosetting variant
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
RD/A	Respiratory distress/metabolic acidosis
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SCF	Stem cell factor
SD	Standard deviation
SDS	Sodium dodecyl sulphate
shRNA	Short hairpin RNA
siRNA	Small or short interfering RNA
SMA	Severe malarial anaemia
TBST	Tris PBS 0.1% Tween

TRIS	Tris(hydroxylmethyl)aminomethane
TU	Transducing viral units
VSA	Variant surface antigen
WHO	World Health Organization
WT	Wildtype

VI. Contents

VII. List of Figures

VIII. List of tables

1. Chapter I: Introduction

1.1 The worldwide burden of malaria

There are over 200 million cases of malaria worldwide every year and at least 435,000 deaths (WHO, 2018) (Figure 1-1). The vast majority of these deaths are due to *Plasmodium falciparum* and 93% occur in African countries, with children under 5 years of age accounting for 61% of the total mortality (WHO, 2018). While great progress has been made over the past decade in reducing the number of cases and deaths, recent World Health Organization (WHO) Malaria Reports warn that “progress has stalled,” and we are “off course” to meet the WHO targets of reducing the morbidity and mortality of malaria worldwide (WHO, 2017, WHO, 2018). There are multiple reasons behind this (WHO, 2018), including parasite resistance to anti-malarial drugs, mosquito resistance to insecticides, reduced funding, and high incidence countries struggling to reduce case numbers (WHO, 2018). In some cases this is due to conflict or challenging political situations, and also the emergence of new threats such as Ebola, which decimated the population of health care professionals in a number of African countries and severely disrupted healthcare infrastructure (WHO, 2015b, Parpia et al., 2016).

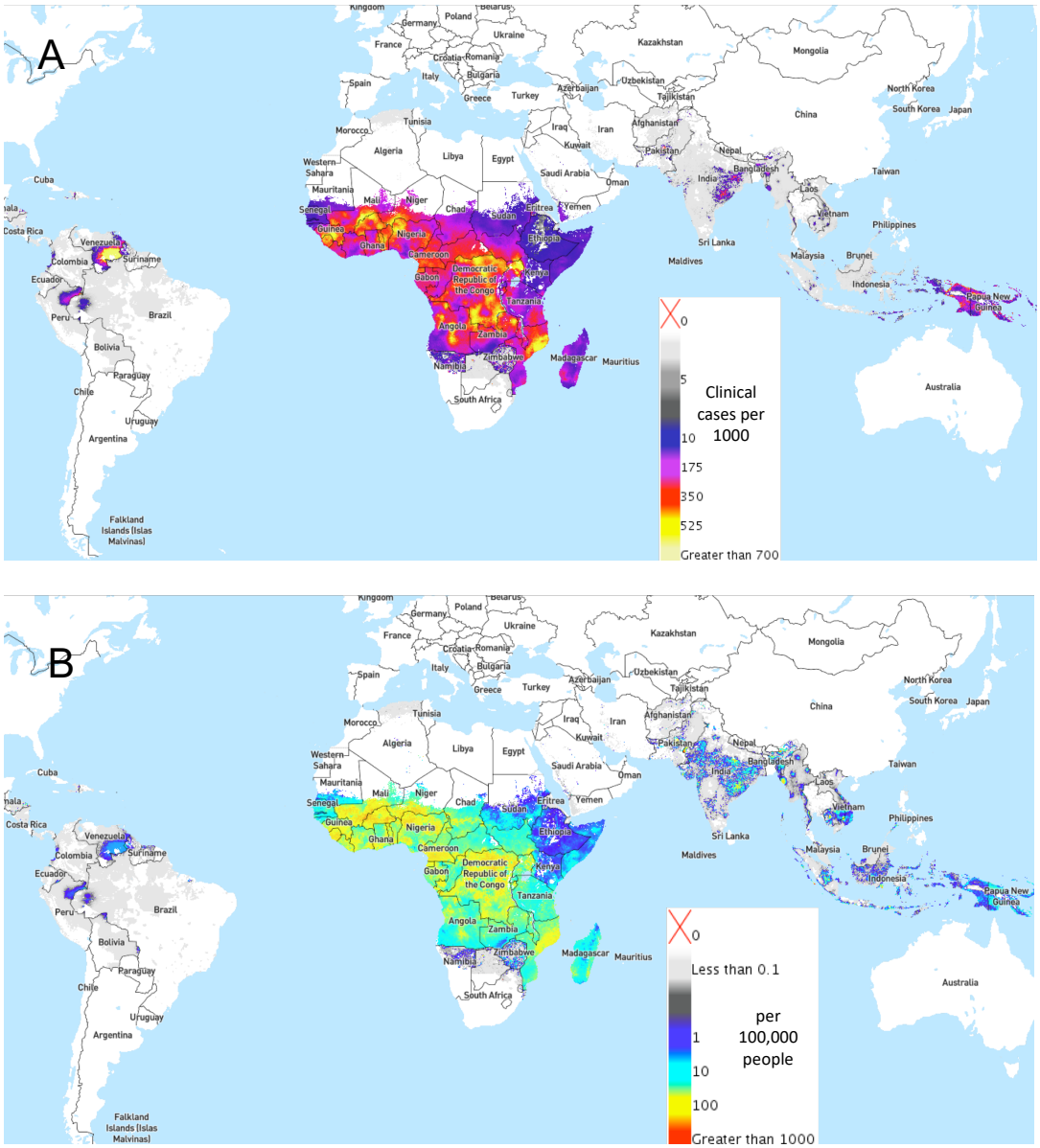


Figure 1-1 The global burden of malaria. A Annual incidence of P. falciparum malaria in 2017, clinical cases per 1000 population. B The mortality of malaria worldwide per 100,000 people. Maps modified from the Malaria Atlas Project, available under the Creative Commons Attribution 3.0 Unported License. http://map.ox.ac.uk.

There are five main *Plasmodium* species which cause disease in humans; *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (Ashley et al., 2018). The latter, *P. knowlesi*, is a zoonotic malaria, the natural host being macaque monkeys, and is of increasing concern particularly in Malaysia and South East Asia where it causes severe disease and death (Singh et al., 2004, Barber et al., 2013, Barber et al., 2017). However, the total number of *P. knowlesi* cases remains low; around 3600 in 2017 (WHO, 2018). After *P.*

falciparum, *P. vivax* contributes most to the clinical burden of disease accounting for around 3.5% of worldwide cases (approximately 7.5 million), though this varies greatly by region from 0.3% of cases in Africa to 74.1% in South America (WHO, 2018). But infection with *P. falciparum* is far and away the greatest cause of malaria related mortality and morbidity worldwide accounting for over 95% of cases and almost all deaths (WHO, 2018). The remainder of this introduction and thesis will therefore concentrate on *P. falciparum*.

1.2 *Plasmodium falciparum* life cycle

The malaria parasite is transmitted by the female *Anopheles* mosquito, which injects sporozoites intradermally during a human blood meal (Figure 1-2) (White et al., 2014). The incubation period from infection to symptoms is roughly 12-14 days, representing 5-8 days in the liver, followed by 6-8 days multiplying through the blood stage (White et al., 2014).

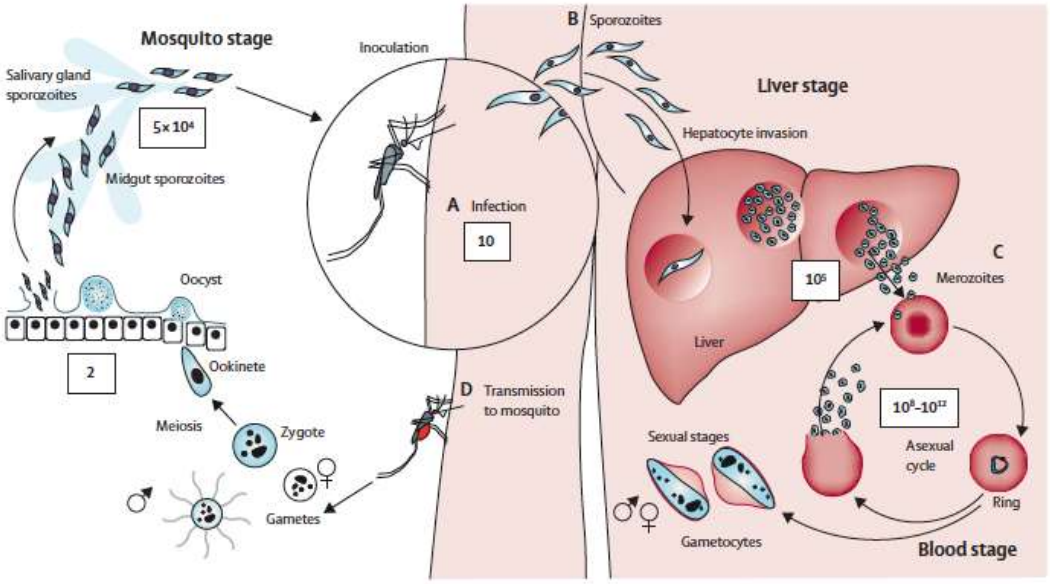


Figure 1-2 The life cycle of P. falciparum, taken from (White et al., 2014).

The sporozoites pass briefly into the blood before invading the hepatocytes of the liver where they multiply to produce around 10,000 merozoites (the

liver stage). The merozoites emerge from the hepatocytes to enter the blood stage where they seek out erythrocytes to invade. There are a number of invasion pathways, including utilizing the host receptors Glycophorin A (Bei et al., 2010), Glycophorin B (Mayer et al., 2009) and Glycophorin C (Maier et al., 2003, Mayer et al., 2006), Complement receptor 1 (Tham et al., 2010), Basigin (Crosnier et al., 2011), CD55 (Egan et al., 2015) and possibly Band 3 (Goel et al., 2003). Once inside the erythrocyte, over the next 48 hours, the merozoite matures (Figure 1-3) through the ring stage, into trophozoites which then undergo schizogony to produce between 8-36 merozoites (Garg et al., 2015). These new merozoites then burst out of the erythrocyte, destroying it, and begin the cycle again with fresh, uninfected erythrocytes.

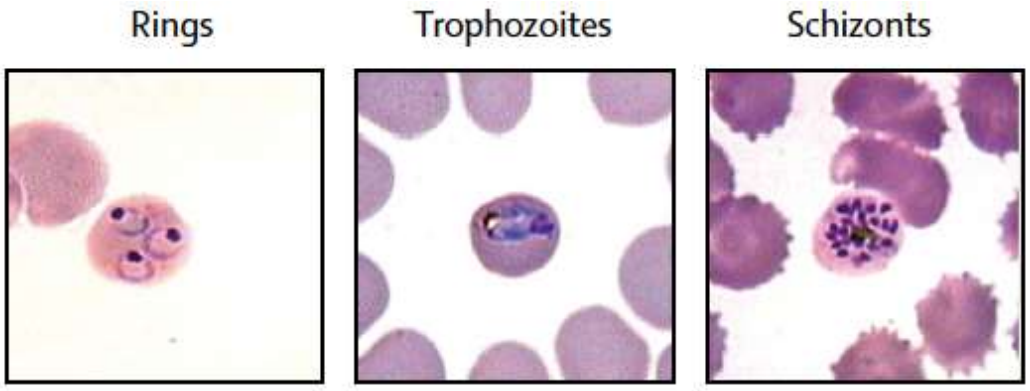


Figure 1-3 Maturation of P falciparum in the erythrocyte. Adapted from (Ashley et al., 2018)

While there are many potential targets for intervention throughout the life cycle of *P. falciparum*, the work contained in this thesis concentrates on the blood stage, specifically after the parasite has invaded the erythrocyte and matured sufficiently to express the parasite derived ligands, PfEMP1, RIFIN and STEVOR (discussed in detail below).

1.3 Clinical presentation and treatment of severe *P. falciparum* malaria

1.3.1 Clinical features of severe malaria

Despite the huge incidence and unacceptably high absolute number of deaths, it is important to note that the majority of people living in endemic countries who become infected with *P. falciparum* malaria do not experience life-threatening disease. Most experience an unpleasant, 'flu-like' illness with fever, chills, muscle aches, headaches and/or diarrhoea (Ashley et al., 2018). The WHO has developed clinical and laboratory definitions of severe malaria, shown in Table 1-1, and there are other scores and techniques, which can further subclassify specific syndromes such as the Blantyre Coma Score (Molyneux et al., 1989) and assessing for malarial retinopathy to diagnose cerebral malaria (Taylor et al., 2004).

Broadly speaking, there are three main syndromes of severe malaria which affect children; cerebral malaria (CM), severe malarial anaemia (SMA) and respiratory distress/metabolic acidosis (RD/A (White et al., 2014, Maitland, 2015, Marsh et al., 1995). In adults, pregnancy-associated malaria and kidney failure are also important (White et al., 2014). Overlap between the syndromes does occur and is predictive of an increase in mortality; a large study of children with severe malaria found 43% of children with a combination of reduced consciousness, acidosis and renal impairment (defined by a blood urea nitrogen >20mg/dl) died, compared to 6-7% of those with only one of these features (von Seidlein et al., 2012).

Table 1-1 Clinical and laboratory feature of severe malaria in children and adults. The abbreviation in bold indicate the associated severe malaria syndrome. Based on (2014) and (WHO, 2012)

	Frequency in children	**Frequency in adults**
Clinical features		
Impaired consciousness (GCS<11, BCS<3) **CM**	Common	Fairly common
Respiratory distress/acidotic breathing **RD/A**	Common	Fairly common
Seizures **CM**	Very Common	Less common
Prostration **CM**	Common	Common
Shock	Less common	Less common
Pulmonary oedema	Unusual	Less common
Abnormal bleeding	Unusual	Less common
Jaundice	Less common	Common
Laboratory features		
Severe anaemia **SMA**	Common (Hb < 5g/dl/Hct <15%)	Less common (Hb < 7g/dl/Hct <20%)
Hypoglycaemia (Glucose <2.2)	Common	Fairly common
Acidosis **RD/A**	Common	Fairly common
Hyperlactataemia **RD/A**	Common	Fairly common
Renal impairment (creatinine > 265μM or urea > 20mM)	Unusual	Common
Hyperparasitaemia (>10%)	Fairly common	Less common
Other features of severe malaria (not part of WHO definition)		
Duration of illness	1-2 days	5-7 days
Resolution of coma	1-2 days	2-4 days
Invasive bacterial co-infection	Common	Uncommon

GCS= Glasgow Coma Score, BCS = Blantyre Coma Score, Hb= haemoglobin, Hct= haematocrit, CM= feature of cerebral malaria, RD/A= feature of respiratory distress/acidosis, SMA= feature of severe malarial aneamia

The incidence of severe malaria is difficult to estimate, in part due to the undocumented cases which never present to healthcare service (WHO

2014), but also due to inter-country variability in whether uncomplicated and/or severe cases are managed as inpatients (Camponovo et al., 2017). Taking into account these 'hidden' cases, the WHO estimates there are around 2 million cases of severe malaria annually, around 1% of the total (WHO 2014). However, this estimate is of all cases, both adult and children, while the incidence specifically in the paediatric population is likely to be much higher. Gonçalves *et al.* (Gonçalves et al., WHO 2014) conducted a large, prospective study following 882 children in Tanzania from birth and found that 81.1% had at least one episode of *P. falciparum* and of these, 11.6% had at least one episode of severe malaria, according to the WHO classification. Interestingly, many of these severe cases occurred after at least one uncomplicated malaria infection, and 15 children had more than one episode of severe disease (Gonçalves et al., WHO 2014). There were 11 malaria related deaths; giving an overall mortality of those who had at least one malaria infection of 1.5%, and for those with severe malaria of around 10% (Gonçalves et al., WHO 2014), similar rates to those seen in other studies of treated severe malaria (Dondorp et al., 2010, Manning et al., WHO 2014, Ndila et al., 2018). The prospective design of this study is particularly useful as many other estimates of severe disease rely on assessing a subset of children who have been admitted to hospital and are therefore likely to be suffering from more severe symptoms. For example, Okiro *et al.* studied clinical admission data from 17 communities in 7 different malaria endemic countries and found that 4% of admitted children were suffering from cerebral malaria, and 17% from severe malarial anemia (Okiro et al., 2009). Earlier estimates by Snow *et al.* demonstrated the influence of different transmission intensities on the clinical presentation of severe malaria in a study comparing a relatively low transmission area, Kilifi in Kenya, with a high transmission area, Ifakara in Tanzania (Snow et al., 1994). Of those children under 10 years of age admitted to a paediatric ward with malaria in Kilifi, 11% had cerebral malaria, and 13% had severe malarial anaemia. By comparison, only 2.8% of children in the Tanzanian hospital had cerebral malaria, but 40% had severe anaemia (Snow et al., 1994). Another hospital based study,

including sites in Kenya and The Gambia, found cerebral malaria rates ranging from 0.7-10% of malarial admission and severe malarial anaemia in 6.7-25% (Snow et al., 1997). Again, these percentages are likely to be overestimates given they are proportions of children already admitted to hospital, nevertheless, these figures illustrate the serious problem of severe malaria in young children.

1.3.2 Management of severe malaria

One of the especially challenging aspects of managing severe malaria, particularly in resource limited setting, is the lack of adjuvant treatments directed against severe forms of the disease (Maitland, 2015). The AQUAMAT study established intravenous Artesunate as the initial treatment of choice for African children with severe malaria (in preference to Quinine) (Dondorp et al., 2010), followed by a course of oral artemisinin-based combination therapy (WHO, 2012). However, despite treatment, around 10% of children with severe malaria still died (Dondorp et al., 2010) and a meta-analysis assessing mortality from severe malaria over the past 30 years has shown minimal improvements in case fatality rates (Manning et al., WHO 2014). In addition to these mortality figures, the morbidity suffered by survivors of severe malaria is significant and often not fully appreciated. Recent reports suggest that over half of children who survive cerebral malaria have some form of developmental, cognitive or behavioral impairment (Langfitt et al., 2019), greater than previously thought (Idro et al., 2010). Adjuvant treatments which have been trialed for severe malaria include steroids (Warrell et al., 1982, Hoffman et al., 1988), anti-inflammatory drugs and aspirin (Hemmer et al., 1991, Di Perri et al., 1995, Looareesuwan et al., 1998, Das et al., 2003, Lell et al., 2010), heparin (Smitskamp and Wolthuis, 1971, WHO, 1986, Hemmer et al., 1991, Rampengan, 1991), monoclonal antibodies and intravenous immunoglobulins (Taylor et al., 1992, van Hensbroek et al., 1996, Looareesuwan et al., 1999), anti-convulsants (White et al., 1988, Crawley et al., 2000, Gwer et al., 2013), N-acetylcysteine

(Watt et al., 2002, Treeprasertsuk et al., 2003, Charunwatthana et al., 2009) and L-arginine (Yeo et al., 2013). None of these treatments have shown consistent benefit and many have even been harmful. Studies have also assessed the role of supportive therapies such as intravenous fluids (Day et al., 2000, Maitland et al., 2003, Maitland and Newton, 2005, Maitland et al., 2005, Akech et al., 2006, Akech et al., 2010, Maitland et al., 2011), and have concluded that very cautious fluid resuscitation is required (Maitland et al., 2011). Blood transfusion does appear to be beneficial (Bojang et al., 1997, Olupot-Olupot et al., WHO 2014) and the ongoing TRACT trial (ISRCTN8408586) may shed more light on the appropriate parameters for transfusion in severe malaria, however the availability of appropriate blood products can be a challenge in some settings.

There is therefore a pressing need to develop a greater understanding of the pathological processes occurring in severe malaria, in order to better inform the development of therapies, specifically directed against this potentially devastating condition.

1.4 The pathogenesis of severe malaria

There are numerous factors, both known and unknown, which influence whether or not a person infected with *P. falciparum* will develop severe or uncomplicated disease. As with any infectious disease, both host and pathogen drivers play an important role, and as yet, our understanding of the pathogenesis of severe malaria is incomplete (Miller et al., 2002, Wassmer et al., 2015, Wassmer and Grau, 2017). For those living in malaria endemic regions, the risk of severe disease falls with increasing age (Figure 1-4) (White et al., WHO 2014) which suggests the development of some form of immunity or disease tolerance, though this does depend on transmission rates (Okiro et al., 2009). However, how this immunity/tolerance develops and what form it takes is unclear; data from Goncalves *et al.* (Gonçalves et al., WHO 2014) demonstrates that many cases of severe disease occur after

several uncomplicated infections; around 10% of those experiencing severe malaria developed this after at least 7 infections (Gonçalves et al., WHO 2014).

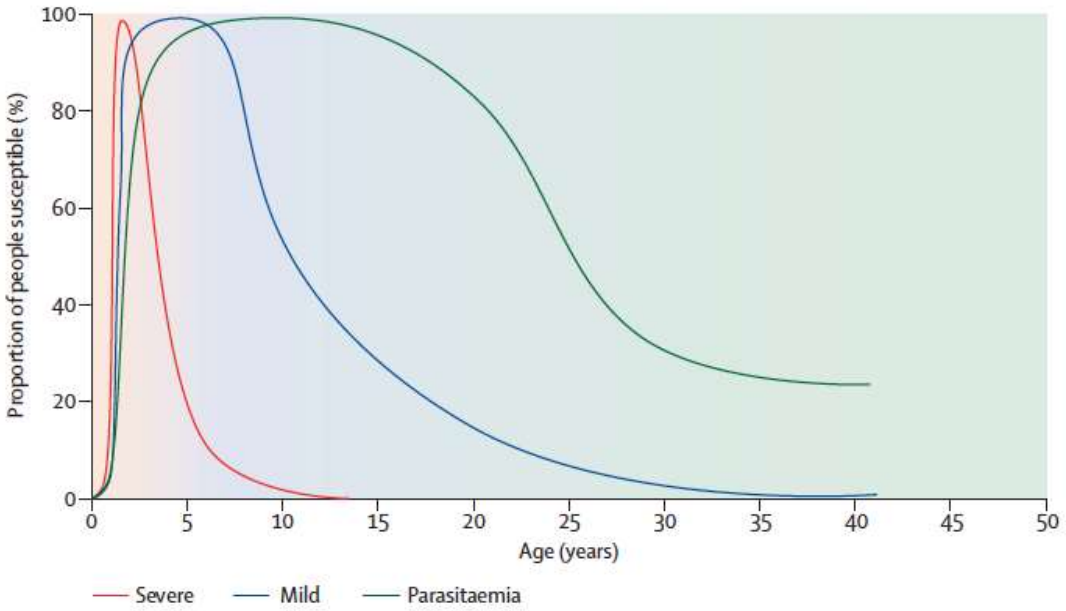


Figure 1-4 Severe malaria decrease with age. Taken directly from (White et al., WHO 2014).

1.4.1 Cytoadhesion in malaria

A key aspect of the pathogenicity of the *P. falciparum* parasites is the ability of infected erythrocytes to adhere to other human cells, such as endothelial cells (sequestration), other erythrocytes (rosetting), platelets (platelet-mediated clumping), or the placenta (pregnancy associated malaria) (Miller et al., 2002, Rowe et al., 2009, Wassmer et al., 2015, Wassmer and Grau, 2017).

1.4.1.1 Sequestration and endothelial cell binding

In *P. falciparum*, erythrocytes infected with mature trophozoite forms of the parasites adhere to endothelial cells in the microvasculature in a process known as sequestration. As a result, mature parasites are protected from removal by the spleen and only erythrocytes containing ring stage parasites

are seen in the periphery (Marchiafava and Bignami, 1894, David et al., 1983, Silamut et al., 1999). Sequestered parasites have been seen at autopsy (Figure 1-5) (Marchiafava and Bignami, 1894, Silamut et al., 1999, Pongponratn et al., 2003, Ponsford et al., 2012, White et al., 2013, Barrera et al., 2018), *in vivo* in the retina (Beare et al., 2009, Barrera et al., 2018) and in the buccal and rectal mucosa (Dondorp et al., 2008a) (Figure 1-6). These sequestered parasites, possibly along with rosetting erythrocytes, cause obstruction of the microvasculature (Figure 1-5) (Silamut et al., 1999, Pongponratn et al., 2003, Dondorp et al., 2004, Dondorp et al., 2008a, Beare et al., 2009, Ponsford et al., 2012), with the level of congestion correlating with the disease severity (Dondorp et al., 2008a, Ponsford et al., 2012). In addition to obstruction, adhesion of the erythrocytes also leads to pathological endothelial cell activation with inflammation, dysregulation of coagulation and breakdown of the barrier function of the endothelium (Moxon et al., 2009, Wassmer et al., 2011, Moxon et al., 2013, Aird et al., WHO 2014, Moxon et al., 2014, O'Sullivan et al., 2016, Wassmer and Grau, 2017)

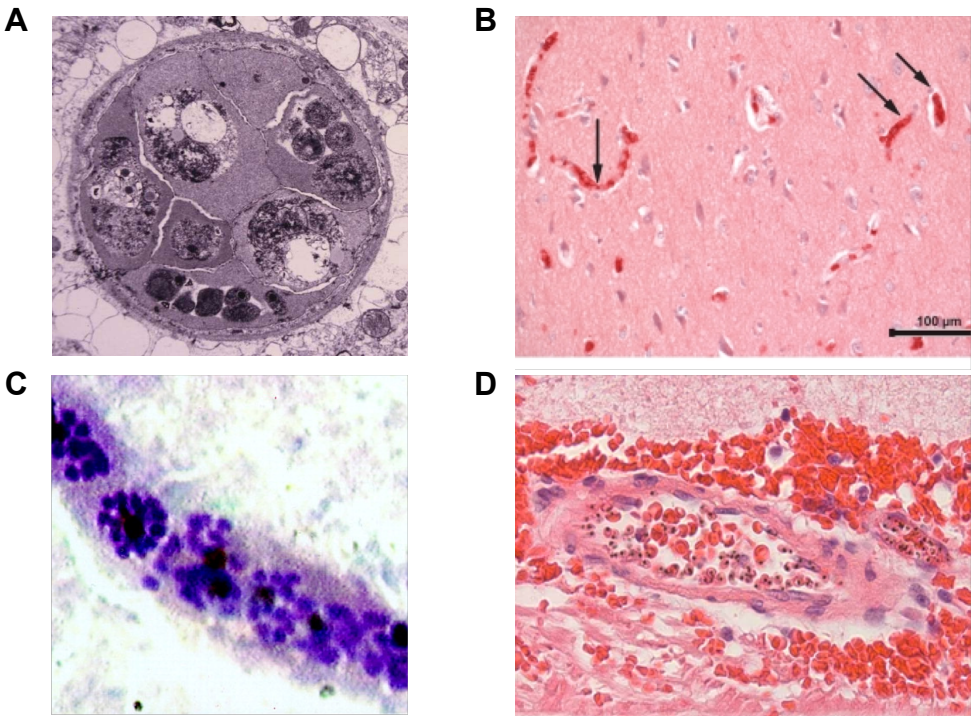


Figure 1-5 Sequestered parasites on postmortem samples. A An electron micrograph of an obstructed cerebral blood vessel full of infected erythrocytes from (Pongponratn et al., 2003). B Obstructed brain microvessels from (Ponsford et al., 2012) C Black stained parasites inside a cerebral vessel (Silamut et al., 1999). D Sequestered parasites in a retinal vessel from (Barrera et al., 2018)

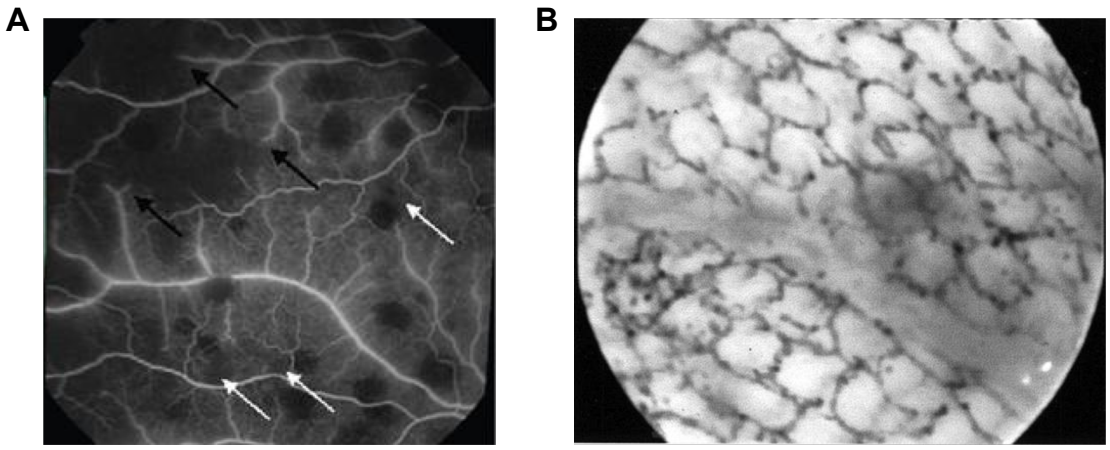


Figure 1-6 A retinal angiogram from (Beare et al., 2009) showing dark black patches of no perfusion and obstructed or "pruned" vessels, identified by black arrows. B Orthogonal polarization spectral image of rectal mucosa showing "cobblestone" congested capillaries containing dark grey erythrocytes from (Dondorp et al., 2008a)

Much work has gone into trying to unpick the mechanisms of endothelial cell binding and identify the relevant parasite ligands and host receptors. In particular, binding to human brain endothelial cells is thought to be key to the pathogenesis of cerebral malaria (Marchiafava and Bignami, 1894, Pongponratn et al., 2003, Ponsford et al., 2012, Barrera et al., 2018, Storm et al., 2019). The important parasite-derived ligands are PfEMP1 (discussed in detail below), particularly the domain cassette (DC) 8 and 13 variants and those encoded by group A or B/A *var* genes (Kyriacou et al., 2006, Avril et al., 2012, Claessens et al., 2012, Lavstsen et al., 2012, Warimwe et al., 2012, Bertin et al., 2013). The corresponding host receptors include CD36, intracellular adhesion molecule-1 (ICAM-1), E-selectin, P-selectin, Thrombospondin, neural cell adhesion molecule (NCAM), platelet endothelial cell adhesion molecule 1 (PECAM-1/CD31), heparan sulfate and endothelial protein C receptor (EPCR) (reviewed in (Rowe et al., 2009, Wassmer and Grau, 2017)). Binding to these host receptors is not necessarily associated with severe disease (Rowe et al., 2009, Turner et al., 2013), with the exception of EPCR which has generated much recent interest (Moxon et al., 2013, Turner et al., 2013, Avril et al., 2016, Jespersen et al., 2016, Storm et al., 2019). In general, recombinant PfEMP1 variants associated with severe disease and clinical isolates from children with severe malaria bind in an

EPCR dependent fashion (Turner et al., 2013, Storm et al., 2019), however results are not consistent throughout all studies (Azasi et al., 2018). In addition, children in Papua New Guinea who survived severe malaria were found to have increased antibodies directed against EPCR-binding PfEMP1 variants in their convalescent serum compared to uncomplicated controls, suggesting that these EPCR-binding parasites contributed to their severe disease (Rambhatla et al., 2019).

1.4.1.2 Rosetting

Rosetting is another important cytoadherence phenotype, specifically associated with severe disease in *P. falciparum* malaria (Rowe et al., 1995, Newbold et al., 1997, Kun et al., 1998, Rowe et al., 2002a, Doumbo et al., 2009, Warimwe et al., 2012). It is usually defined as the adhesion of two or more uninfected erythrocytes to an erythrocyte infected with a mature, asexual parasite (Figure 1-7). Rosetting is thought to contribute to the sequestration of infected erythrocytes in the microvasculature which in turn causes obstruction, inflammation and tissue damage as illustrated above (Kaul et al., 1991, Miller et al., 2002, White et al., 2013). In Africa, rosetting has been linked with all types of severe malaria including cerebral malaria (Rowe et al., 1995, Kun et al., 1998, Doumbo et al., 2009), severe anemia (Newbold et al., 1997) and respiratory distress (Warimwe et al., 2012), and rosettes have been seen at autopsy in the brain (Dondorp et al., 2004). All the human-infecting malaria species rosette (Udomsanpetch et al., 1995, Angus et al., 1996, Chotivanich et al., 1998, Lowe et al., 1998), and while the link between severity of disease and rosetting seems to be confined to *P. falciparum*, there is some evidence that rosetting may contribute to severe anemia in *P.vivax* (Marín-Menéndez et al., 2013). The remainder of this introduction will focus on the role of rosetting in severe malaria and the proposed mechanisms of rosetting in *P. falciparum*.

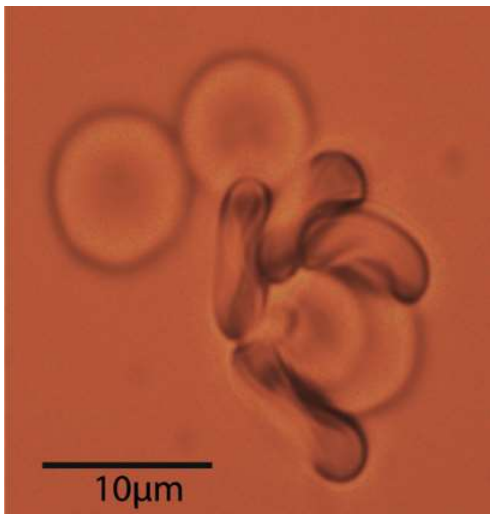


Figure 1-7 A P. falciparum rosette (parasite line ITvar60 in static culture). Image shows an infected erythrocyte and four adherent uninfected cells. Images taken using a Yenway microscope camera on a Leica DM KB2 florescent microscope.

1.5 Rosetting and severe malaria

1.5.1 Evidence for the association between rosetting with severe malaria

Much of the evidence that rosetting is associated with severe malaria comes from field studies in which *P. falciparum* clinical isolates from patients with varying degrees of malaria severity were cultured for 24 hours and the rosette frequency (usually defined as the percentage of 200 infected erythrocytes which are rosetting with 2 or more uninfected erythrocytes) counted and compared (Carlson et al., 1990, Treutiger et al., 1992, Ringwald et al., 1993, Rowe et al., 1995, Newbold et al., 1997, Kun et al., 1998, Heddini et al., 2001, Pain et al., 2001, Rowe et al., 2002c, Doumbo et al., 2009) (Figure 1-8). For example, in Mali, Doumbo *et al.* (Doumbo et al., 2009) found that 90% of isolates from children with severe malaria were capable of rosetting, with a median rosette frequency of 20%, compared to 55% of isolates from uncomplicated cases and these parasites only had a median rosette frequency of 1%. These findings have been replicated in multiple different populations; in Kenya (Rowe et al., 1995, Newbold et al.,

1997, Heddini et al., 2001, Pain et al., 2001, Rowe et al., 2002c), Mali (Doumbo et al., 2009), Uganda (Normark et al., 2007), Madagascar (Ringwald et al., 1993), the Gambia (Carlson et al., 1990, Treutiger et al., 1992), Gabon (Kun et al., 1998) and a hyperendemic region of India (Rout et al., 2010) the rosetting frequency was higher in patients, usually children, with severe malaria. However, studies from Papua New Guinea (al-Yaman et al., 1995) and Malawi (Rogerson et al., 1999) found no significant differences. In Southeast Asia, the picture is less clear; adults in Thailand with severe malaria had similar rosetting rates to those with uncomplicated malaria (Angkasekwinai et al., 1998, Chotivanich et al., 2004). However, rates were higher in one cerebral malaria group (Chotivanich et al., 2004), and it is important to bear in mind the epidemiological differences between African and Asian malaria in terms of age, incidence, transmission intensity and mortality (Dondorp et al., 2008b, Rowe et al., 2009, WHO, 2018).

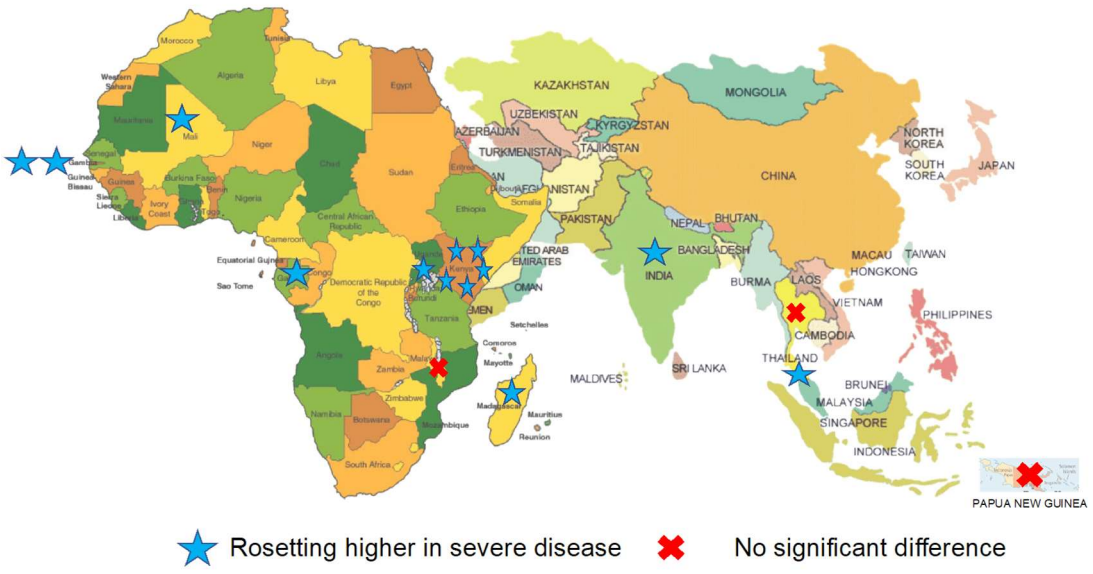


Figure 1-8 Countries in which the association between rosetting and severe disease has been studied. Blue stars show countries in which an association with increased rosetting and severe disease has been demonstrated with multiple stars indicating the number of independent studies, red crosses show countries in which studies have found no significant difference between rosetting rates in severe and uncomplicated disease. Note, map is not to scale and some countries have been overlaid to emphasise the relevant malaria endemic countries.

Other evidence supporting the role of rosetting in the pathology of severe malaria has been found by examining the sera of children at different ages or

with varying severities of malaria. Anti-rosetting antibodies are found more commonly in children with mild, rather than severe, malaria (Carlson et al., 1990, Treutiger et al., 1992, Vigan-Womas et al., 2010) and the ability of sera from children living in malaria endemic areas to disrupt rosettes increases with age, in keeping with the age-related decline in both incidence and mortality (Barragan et al., 1998). Of note, erythrocytes from patients with conditions which provide natural protection against severe malaria, such as sickle cell trait, Thalassemia, and blood group O form smaller, weaker rosettes (Carlson et al., 1994, Barragan et al., 2000b, Opi et al., 2014).

1.5.2 Why do parasites form rosettes?

The benefit to the parasite of rosetting is not entirely clear, as causing severe disease or death of the host is certainly not in its best interests. Plausible suggestions include that the formation of rosettes may shield the infected cell from the host immune system, or that close contact with uninfected erythrocytes could enhance invasion (Wahlgren et al., 1989). Some adhesion phenotypes do provide shielding from the immune system, such as the PfEMP1 variant VAR2CSA, associated with placental binding in pregnancy associated malaria, which utilizes IgM to mask IgG binding sites (Barfod et al., 2011), however there is a lack of evidence for this in rosetting parasites. Erythrocytes infected with rosetting parasites do bind non-immune IgM (Scholander et al., 1996, Rowe et al., 2002c), however this does not appear to protect from IgG mediated phagocytosis (Stevenson et al., 2015a). In addition, merozoites released from rosetting infected erythrocytes do not appear to be protected and invasion rates are not enhanced (Deans and Rowe, 2006). There may be some specific scenarios where rosettes provide an element of antibody shielding (Moll et al., 2015) for example; parasites which show a rosetting preference for blood group A are able to form larger and stronger rosettes when cultured in this blood group (Rowe et al., 1995, Rowe et al., 2007). Two such parasite lines, FCR3S1.2 and PAvarO, showed significantly less rosette disruption by anti-PfEMP1 antibodies when grown in

blood group A compared to blood group O, though this was not seen in parasite strain R29, and invasion efficiency was not increased in the presence of larger rosettes (Moll et al., 2015).

Invasion is also not increased in parasites selected for rosetting compared to non-rosetting parasite from the same clone in various laboratory-adapted strains (Clough et al., 1998b, Deans and Rowe, 2006, Ribacke et al., 2013) and, somewhat surprisingly, merozoites show no preference for invading adjacent uninfected cells contained within the rosette (Clough et al., 1998b). In field isolates, one study on African clinical samples found no correlation between rosette frequency and parasite multiplication rate *in vitro* (Deans et al., 2006), though another suggested there was increased multiplication in rosetting isolates (Ribacke et al., 2013). Similarly, the correlation between parasitaemia and rosetting is not straightforward, Carlson *et al.* (Carlson et al., 1990) found no relationship between rosetting and parasitaemia in the Gambia, while Rowe *et al.* (Rowe et al., 2002a) found that for Kenyan children with severe malaria (but not uncomplicated malaria), there was a positive correlation between rosetting frequency and parasitaemia. *In vivo* studies using splenectomized *Saimiri sciureus* monkeys demonstrated a 1.5 times higher parasite multiplication with rosetting compared to non-rosetting parasites (Le Scanf et al., 2008) therefore more work is required to determine the contribution of rosetting in promoting invasion or reducing parasite clearance.

Rosetting may have a role in aiding sequestration, thereby avoiding splenic clearance of the infected erythrocytes and contributing to microvascular obstruction (Kaul et al., 1991). Rosetting parasites of the line IT/R29 are also capable of binding human brain endothelial cells (Adams et al., 2014) and antibodies to the NTS-DBL1α or DBL2γ domains of the IT4var09 PfEMP1 variant were able to reverse this adhesion. Both these domains were also found to bind heparin, and heparin was also capable of disrupting adhesion to the endothelial cells (Adams et al., 2014). While heparin and NTS-DBL1α

antibodies are also able to disrupt rosettes (Rowe et al., 1994, Ghumra et al., 2011), antibodies to the DBL2γ domain had no effect on rosette frequency (Ghumra et al., 2011) suggesting a different, but not mutually exclusive, host receptor-parasite ligand interaction for these two different cytoadhesion phenotypes (Adams et al., 2014).

Therefore, while a role for rosetting in promoting invasion, immune evasion and sequestration is plausible, the evidence for each is incomplete and further studies are required to determine both the advantage which rosetting affords to the parasites, and the exact mechanism by which rosetting contributes to severe disease.

1.6 Mechanisms of rosetting: Parasite derived rosetting ligands

As alluded to above, there are two sides to consider when investigating the molecular mechanisms of rosetting in *P. falciparum*; the parasite derived ligands and the corresponding host receptors. While the work of this thesis concentrates mainly on the host receptors, a summary of the important parasite derived ligands is important for context.

1.6.1 PfEMP1

Of the three variant surface antigen (VSA) families involved in rosetting, the most well established is *Plasmodium falciparum* Erythrocyte Membrane Protein One (PfEMP1). PfEMP1 variants are found on the cell surface of infected red blood cells, first appearing around 18-24 hours after invasion (Chen et al., 1998, Kraemer and Smith, 2006, Smith et al., 2013, Hviid and Jensen, 2015), and are encoded by *var* genes (Su et al., 1995, Hviid and Jensen, 2015). Usually only one *var* gene is transcribed at a time per cell, though exceptions to this have been described (Joergensen et al., 2010). There can be switching of transcription between around 60 *var* genes per

parasite isolate with minimal overlap between parasite strains (Roberts et al., 1992, Su et al., 1995, Scherf et al., 1998, Kraemer and Smith, 2006). This switching can be fast, as often as every cycle (Bachmann et al., 2011), and is highly coordinated *in vivo* though this synchronization may be lost with prolonged *in vitro* culture (Bachmann et al., 2011). This high level of antigenic variation makes PfEMP1 a challenging vaccine target.

1.6.1.1 Var genes

Var genes consist of two exons; exon 1 encodes the multiple Duffy binding-like (DBL) domains and cysteine-rich interdomain regions (CIDR) which make up the extracellular part of the protein, while exon 2 encodes the intracellular domain (Hviid and Jensen, 2015) (Figure 1-9). In general, *var* genes can be grouped based on location and upstream promoter sequence as A/upsA, B/upsB or C/upsC, though intermediate groups (B/A and B/C) do exist (Gardner et al., 2002, Lavstsen et al., 2003, Kraemer and Smith, 2006). An additional gene, *var2CSA*, sometimes classified as UpsE, codes for the highly conserved chondroitin sulfate-binding PfEMP1 specific to pregnancy associated malaria (Salanti et al., 2003). Group A *var* genes, are subtelomeric and transcribed towards the telomere, group B are also subtelomeric but transcribed in the opposite direction, while Group C are internal (Gardner et al., 2002, Lavstsen et al., 2003). PfEMP1 encoded by Group A *var* genes are larger, consisting of five or more extracellular domains (as shown in Figure 1-9) and do not bind CD36 (Robinson et al., 2003) while those from Group B and C *var* genes have only 4 domains and are able to bind CD36 (Lavstsen et al., 2003, Robinson et al., 2003). Importantly, particularly for vaccine design, PfEMP1 from Group A *var* gene are associated with severe disease, while Group C are not (Rottmann et al., 2006, Kaestli et al., 2006, Kyriacou et al., 2006, Falk et al., 2009, Warimwe et al., 2009, Warimwe et al., 2012, Turner et al., 2013, Tembo et al., 2014). The association is less clear for Group B *var* genes, though the intermediate group B/A may be linked to severe disease (Kaestli et al., 2006, Kyriacou et al., 2006, Rottmann et al., 2006, Turner et al., 2013, Tembo et al., 2014).

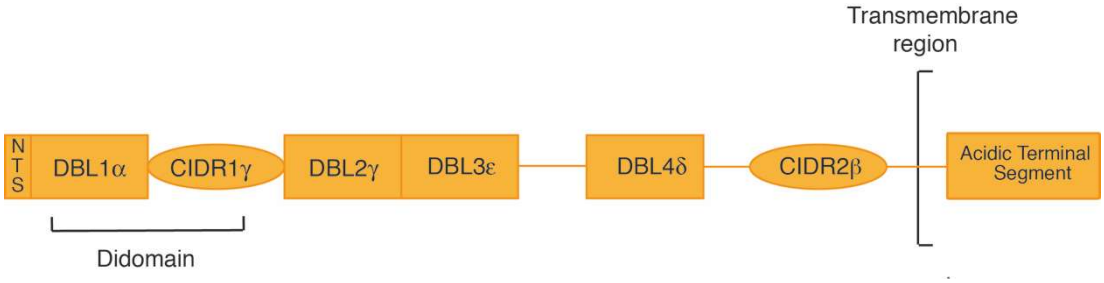


Figure 1-9 PfEMP1 of ITvar9 adapted from (Ghumra et al., 2011). NTS= N-terminal sequence, DBL= Duffy binding-like domain, CIDR= cysteine-rich interdomain regions.

1.6.1.2 PfEMP1 structure

The PfEMP1 protein has a molecular weight of around 200-450kDa, made up of the highly variable extracellular domains and an intracellular acidic terminal segment (Su et al., 1995, Smith et al., 1995, Baruch et al., 1995). The cytoplasmic region of PfEMP1 interacts with knob-associated histidine-rich protein (KAHRP) leading to clusters of PfEMP1 on tiny projections known as knobs (Figure 1-10) (Berendt et al., 1994, Waller et al., 1999, Kilejian, 1979, Aikawa, 1988). Some lines, such as ITvar60, are knobless, though the lack of knobs leads to a decrease in overall PfEMP1 expression and reduced adhesive capacity (Horrocks et al., 2005, Subramani et al., 2015).

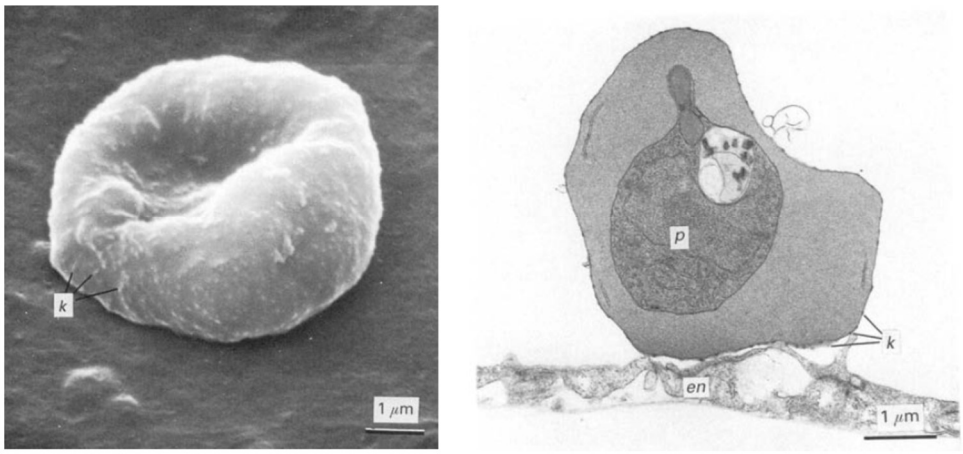


Figure 1-10 Scanning (LEFT) and transmission (RIGHT) electron micrographs of infected erythrocytes displaying knob protrusion (labelled k). Both infected erythrocytes are adherent to a human umbilical endothelial cells (en). Taken from (Berendt et al., 1994).

The extracellular part of the molecule is made up of various DBL and CIDR domains which can be classified as DBL α, β, γ, δ, ε, ζ, PAM, and CIDR α, β, γ, δ and PAM respectively (Rask et al., 2010). The domains can be further subdivided numerically, for example DBLα1.1 or CIDRα1.4, based on sequence similarity (Rask et al., 2010). When certain combinations of PfEMP1 domains are frequency seen together, these are known as 'domain cassettes' (DC), for example DC8 is made up of DBLα2, CIDRα1.1, DBLβ12 and DBLγ4/6, and DC13 is made up of DBLα1.7 and CIDRα1.4 (Rask et al., 2010). Both these domain cassette variants are of particular interest as the they bind human brain endothelial cells and have been linked to severe malaria (Avril et al., 2012, Claessens et al., 2012, Lavstsen et al., 2012, Turner et al., 2013).

Not all PfEMP1 variants are capable of rosetting, however the rosetting variants for a number of parasites lines have been identified, as shown in Figure 1-11(Vigan-Womas et al., 2011, Ghumra et al., 2012). In terms of rosetting, the DBL1α domain is thought to be most important (Rowe et al., 1997, Russell et al., 2005, Vigan-Womas et al., 2008), though other domains can bind uninfected erythrocytes (Rowe and Donat personal communication and Chapter 3). Antibodies raised against various domains of PfEMP1

disrupt rosettes, however, perhaps unsurprisingly given the very variable PfEMP1 repertoire, these antibodies do not cross react across all strains (Ghumra et al., 2011, Ghumra et al., 2012), a key requirement for an effective anti-rosetting vaccine.

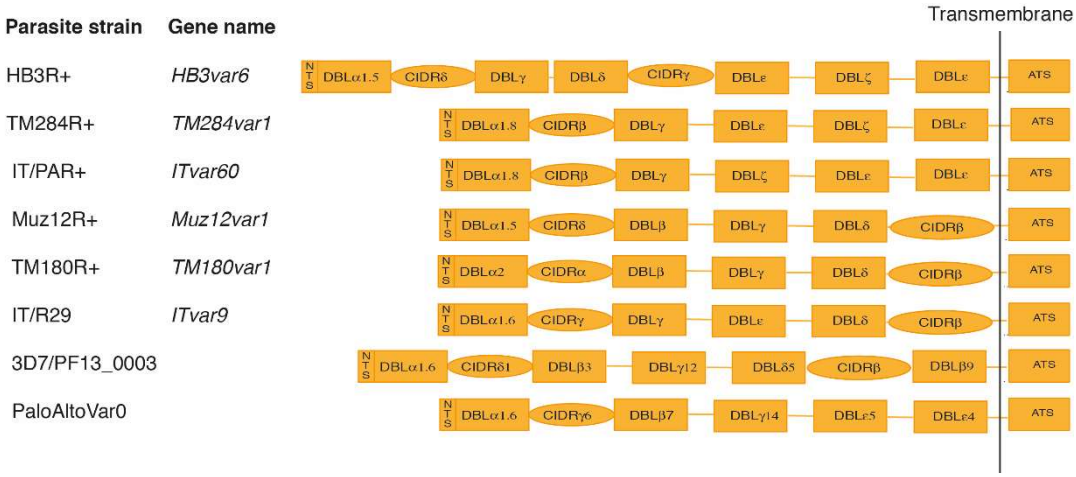


Figure 1-11 Rosetting PfEMP1 variants. Data from (Ghumra et al., 2012) and (Vigan-Womas et al., 2011)

1.6.2 Other molecules: RIFINS and STEVOR

Recent evidence has renewed interest in both the repetitive interspersed families of polypeptides (RIFIN) and proteins encoded by sub-telomeric variant open reading frame (STEVOR) genes (Yam et al., 2017). Both RIFIN (Kyes et al., 1999, Goel et al., 2015) and STEVOR (Bachmann et al., 2015) are variant surface antigens also found on the membrane of infected erythrocytes. RIFINs are relatively trypsin resistant and earlier work had shown that removal of PfEMP1 from ITvar60 infected erythrocytes with low concentrations of trypsin greatly reduced rosetting frequency but did not abolish it completely (Kyes et al., 1999), indicating that RIFINs could have an accessory function. More recently, it has been suggested that RIFINs have a particular role in rosetting in blood group A (Goel et al., 2015). Experiments showed higher rosetting rates in parasites cultured in blood group A compared to O after removal of PfEMP1 with trypsin, higher binding of group A erythrocytes to RIFIN transfected CHO cells and increased rosetting rates

in parasites transfected with a *rif* gene, again only in blood group A (Goel et al., 2015). STEVOR proteins have been shown to bind Glycophorin C on uninfected RBCs and anti-STEVOR antibodies disrupt rosettes in some strains (Niang et al., 2014). Recent data have suggested that STEVOR may have a role in strengthening rosettes, particularly in later stage trophozoites (Singh et al., 2016).

1.7 Mechanisms of rosetting: Host rosetting receptors

Given the huge antigenic variance of the parasite-derived rosetting molecules described above, it is vital to identify the corresponding host erythrocyte receptors in order to investigate potential alternative therapeutic options. A number of receptors have been proposed (Figure 1-12) which are discussed in detail below and in Table 1-2, however none offers a full explanation for all parasite strains, and it is likely that different receptors can be utilized by various parasites at different times. In addition, some receptors may be essential whereas others may be neither necessary nor sufficient but have a role in strengthening rosettes.

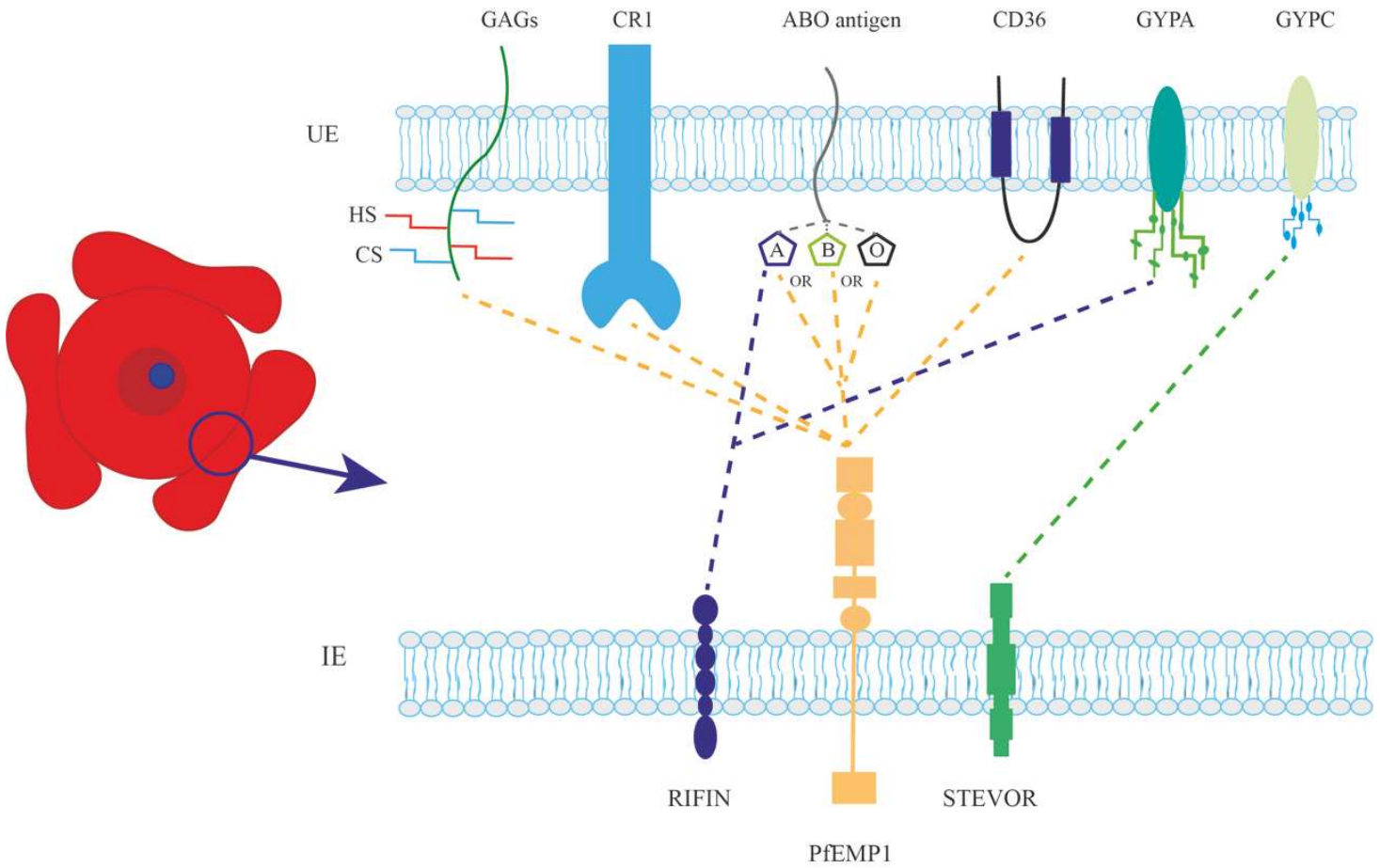


Figure 1-12 Parasite derived ligands and host receptors involved in rosetting UE= uninfected erythrocyte, IE= infected erythrocyte, GAGs= glycosaminoglycans, HS= heparan sulfate, CS= chondroitin sulfate, CR1= complement receptor 1, GYPC = glycophorin C, GYPA = glycophorin A. Dashed lines represent proposed host receptors for each parasite ligand. From (McQuaid & Rowe, 2019 submitted)

1.7.1 ABO blood group

Arguably the most clinically important blood group antigens are those of the ABO system which result in 4 different blood groups; A, B, AB and O (Dean, 2005). The A and B antigens differ based on the sugar at the antigen terminus; for blood group A this is N-acetyl-D-galactosamine and for B, D-galactose (Dean, 2005). Those with blood group AB have both antigens, while blood group O erythrocytes express only the H antigen, a precursor to the A and B antigens (Dean, 2005). Blood group O is known to protect against severe malaria as demonstrated by multiple studies across Africa (Rowe et al., 2007, Tekeste and Petros, 2010, Malaria Network, 2014, Degarege et al., 2019) and India (Rout et al., 2012). A recent meta-analysis by Degarege *et al.* (Degarege et al., 2019) found that the odds of severe disease were significantly higher for blood groups A,B and AB compared to O. Overall they also concluded that blood group does not appear to have any effect on parasitaemia or haemoglobin level, though there was some heterogeneity between the individual studies included in the meta-analysis (Degarege et al., 2019). Interestingly, Theron *et al.* (Theron et al., 2018) tested multiple donors of different blood groups and found that parasites preferentially invade blood group O erythrocytes. This was a surprising finding, however due to the single cell flow cytometry gating strategy used, it is possible that the authors unintentionally excluded erythrocytes in rosettes from their analysis.

The proposed mechanism for the protection against severe malaria seen for blood group O is through reduced rosetting with this blood type (Udomsangpetch et al., 1993, Cserti and Dzik, 2007, Rowe et al., 2007). Parasites cultured from patients of different blood groups show significant variation in rosetting ability; samples from patients with blood group O rosette significantly less than those from blood group A, B or AB (Rowe et al., 1995, Rowe et al., 2007, Rout et al., 2012). The differences in median rosette frequency are relatively low, (rosette frequency 2 vs 7% for O vs A in Kenya

(Rowe et al., 1995), approximately 5 vs 15% in Mali (Rowe et al., 1995, Rowe et al., 2007) and approximately 17 vs 27% in India (Rout et al., 2012)). These figures do not necessarily reflect the size of the rosettes, which are often bigger in non-O blood groups (Barragan et al., 2000b) and may therefore contribute more towards microvascular obstruction and the observed differences in rosette frequency do correlate with protection against severe malaria (Rowe et al., 2007, Rout et al., 2012). Of note, blood group O only appears to protect against severe disease in the context of rosetting parasites (Rowe et al., 2007); group O patients infected with non-rosetting parasites were no more protected against severe disease than other blood groups, adding further evidence to the hypothesis that this protection is due to a reduction in rosetting.

In vitro studies culturing the same parasite in different blood groups have also confirmed that many parasites show a rosetting preference for blood groups A or B rather than O (Carlson and Wahlgren, 1992, Udomsangpetch et al., 1993, Barragan et al., 2000b, Pipitaporn et al., 2000). When cultured in their preferred blood group, these parasites form stronger, larger rosettes which are more resistant to disruption (Carlson and Wahlgren, 1992, Barragan et al., 2000b, Ch'ng et al., 2016), although the absolute rosette frequency (percentage of infected erythrocytes forming rosettes) does not change. Other studies have also shown that removing the A or B antigens with α-N-acetyl-galactosaminidase decreases the rosette size (Barragan et al., 2000b).

For the group A preferring parasite, Palo Alto 89F5 VarO, the group A antigen binding site has been mapped to specific residues in the NTS-DBLα domain of PfEMP1-VarO and binding is enhanced by the addition of the CIDR1γ domain (Vigan-Womas et al., 2012). The NTS-DBLα domain bound more efficiently to blood group A erythrocytes, followed by B then O, and this binding was related to the amount of blood group A antigen expressed (Vigan-Womas et al., 2012). As discussed above, Goel *et al.* (Goel et al.,

2015) have suggested that RIFINs also bind to the blood group A antigen, which could account for the larger, stronger rosettes seen in this blood group.

Therefore, different blood groups do seem to have a significant and clinically relevant effect on rosetting and susceptibility to severe malaria. However, given rosettes can form in blood group O, it seems likely that ABO blood group variations may have more of a role in strengthening and enlarging rosettes rather than being essential for rosetting.

1.7.2 Complement receptor 1

Complement receptor 1 (CR1) is a membrane glycoprotein which helps prevent damage to self by regulating the complement system (Stoute, 2011, Schmidt et al., 2015). There are a number of CR1 polymorphisms coding for differences in copy number on the cell membrane, molecular weight and sequence, including the Knops blood group system (Stoute, 2011). In the context of malaria, CR1 is thought to be involved in rosetting, complement regulation and invasion (Schmidt et al., 2015), though the association with disease severity varies between different studies and populations (Rowe et al., 2000, Nagayasu et al., 2001, Sinha et al., 2009).

In laboratory based studies, red cells which are deficient in CR1 (i.e. express fewer than 100 molecules per cell (Moulds et al., 1992)) show reduced rosetting and soluble CR1/CR1 antibodies inhibit rosetting (Rowe et al., 1997, Lee et al., 2014). The region of CR1 essential for complement receptor related rosetting has been mapped to the C3b binding site on the long homologous repeat regions (LHR) B and C, and a monoclonal antibody which recognizes these sites, J3B11, reversed rosettes *in vitro* in the parasite clone R29 (Rowe et al., 2000). When other parasite strains and field samples were investigated, J3B11 reversed rosettes in 3 out of 4 laboratory strains, 14 of 15 Kenyan and 5 of 10 Malawian clinical isolates, though the reduction

in rosette frequency was less in the Malawian samples compared to those from Kenya (Rowe et al., 2000).

In keeping with the diversity between isolates from different countries in the laboratory studies, results from population field studies also vary. CR1 deficiency is very common in malaria endemic areas such as Papua New Guinea (Cockburn et al., 2004) and some parts of India (Sinha et al., 2009). There is less information from Africa, where the commonly studied *Hind*III restriction fragment length polymorphism does not correlate with CR1 copy number (Rowe et al., 2002b), however CR1 deficiency seems to be rare in Mali and Kenya (Rowe et al., 2002b, Opi et al., 2016). In a case-control trial in Papua New Guinea, those who were heterozygous for the CR1 low expression allele were protected against severe malaria compared to those homozygous for high expression, but the results for those homozygous for the low expression alleles were not statistically significant (Cockburn et al., 2004). In India, low CR1 levels protected against severe malaria in endemic regions (Sinha et al., 2009, Rout et al., 2011, Panda et al., 2012), but predisposed to severe disease in areas of low malaria incidence (Sinha et al., 2009). The transmission intensity of malaria seems to be particularly important in determining whether high or low CR1 is beneficial as studies from Thailand, where transmission is relatively low compared to Africa, have also shown that low CR1 may be detrimental (Nagayasu et al., 2001, Teeranaipong et al., 2008).

In addition to the association with CR1 copy number, there is evidence to suggest that polymorphisms in the Knops blood group antigens carried on CR1 may also affect susceptibility to severe disease. The Knops blood group consists of antithetical antigen pairs Swain-Langley 1 and 2 (Sl1 and Sl2) and McCoy a and b (McC^a and McC^b) (Moulds, 2010). Rowe *et al.* showed that erythrocytes of the Swain Langley 2 (Sl2) genotype had less binding to COS cells expressing a rosetting PfEMP1-DBL protein (Rowe et al., 1997), though another study found no difference in the ability of CR1 protein

fragments of various Swain-Langly and McCoy genotypes to disrupt rosettes (Tetteh-Quarcoo et al., 2012). More recently, a large case-control study of Kenyan children found that the *Sl2* allele was associated with protection against all severe malaria, cerebral malaria and death, while *McCb* gave an increased risk of cerebral malaria (Opi et al., 2018). The same study also found that isolates from Malian children with the *Sl2/Sl2* genotype had significantly lower rosetting *ex-vivo* than those from children with *Sl1/Sl1* or *Sl1/Sl2*, though no differences were found between the McCoy genotypes (Opi et al., 2018).

More work is therefore required to investigate the links between CR1, rosetting and malaria severity. The laboratory studies suggest it may have an important role to play in rosetting, which could explain the significance of CR1 level in areas of high malaria transmission where rosetting is most strongly associated with severe disease. However, the potential beneficial effects of CR1 as a complement regulator modifying the host response to malaria may be more relevant in the low transmission setting (Stoute, 2011) and this could have implications for the development of CR1 modifying therapies.

1.7.3 Glycosaminoglycans

The glycosaminoglycans, heparan sulfate (HS) and chondroitin sulfate (CS) play a part in multiple stages of the malaria life-cycle, including hepatic invasion for HS (Frevert et al., 1993), cytoadhesion (Vogt et al., 2003, Adams et al., 2014) and, for CS, placental sequestration (Ataíde et al., 2014). Chapter 3 of this thesis deals extensively with the role of these HS and CS in rosetting and a detailed overview of these molecules is given in the introduction to this Chapter 3. Briefly, many studies have used heparin, a highly sulfated form of HS found only in mast cells, as an analogue for HS. These studies found that heparin is effective at disrupting rosettes in many *P. falciparum* strains (Udomsangpetch et al., 1989, Carlson et al., 1992, Rowe

et al., 1994, Barragan et al., 1999), however the mechanism of this disruption is not clear and may not be specific. Importantly, there is only one paper suggesting that HS is indeed present on the surface of mature erythrocytes (Vogt et al., 2004), and I have been unable to replicate these findings (Chapter 3: Results). A further exploration of the role of HS and CS in rosetting is given in subsequent chapters.

1.7.4 CD36

The scavenger receptor, CD36, is widely expressed and has been shown to bind PfEMP1 (Baruch et al., 1996, Chen et al., 2000, Robinson et al., 2003, Cabrera et al., 2014), however these CD36 binding PfEMP1 variants tend to be encoded by *var* genes of the UpsB and C group which are generally associated with mild disease (Robinson et al., 2003, Kraemer and Smith, 2006, Kyriacou et al., 2006). Anti-CD36 antibodies are capable of disrupting rosettes in a single laboratory adapted strain, Malayan Camp (Handunnetti et al., 1992b), but not in any other tested laboratory-adapted or Kenyan clinical isolates (Udomsangpetch et al., 1989, Wahlgren et al., 1992, Rowe et al., 2000, Niang et al., 2014). While CD36 deficiency is fairly common in African populations (Fry et al., 2009), large-scale genetic studies have failed to find any CD36 polymorphisms that are associated with protection against severe malaria (Fry et al., 2009). In fact, CD36 may have an important role to play in innate immunity and platelet associated parasite death (McGilvray et al., 2000, McMorran et al., 2012, Cabrera et al., 2014). It is therefore remains unlikely that CD36 is a clinically significant rosetting receptor or a useful therapeutic target (Cabrera et al., 2014).

1.7.5 Glycophorin A (GYPA, CD235a)

Glycophorin A (GYPA) is highly abundant on erythrocytes and is one of the few molecules to be identified as important for protection against severe malaria by genome wide association studies (Band et al., 2015, Leffler et al.,

2017). GYPA is an invasion receptor for *P. falciparum* through an interaction with the parasite ligand Erythrocyte Binding Antigen 175 (EBA-175) (Sim et al., 1994) and individuals who express a rare hybrid form of Glycophorin A and B, the Dantu blood group, are protected from severe disease, potentially through reduced invasion (Band et al., 2015, Leffler et al., 2017, Ndila et al., 2018, Field et al., 1994). There is some limited evidence to suggest that the GYPA molecule may be involved in rosetting (Goel et al., 2015). Goel *et al.* found that FCR3S1.2 parasites transfected with a RIFIN gene rosetted poorly with GYPA null erythrocytes of blood group O and B, but were still able to rosette normally with GYPA null blood group A erythrocytes, which they concluded was due to the superior effect of RIFIN-Blood group A binding (Goel et al., 2015). However, GYPA monoclonal antibody fragments did not disrupt rosettes in clinical isolates (Lee et al., 2014) or the lab strain 3D7 5A-R+ (Niang et al., 2014) and Rowe *et al.* did not detect a reduction in rosetting in MkMk (GYPA and GYPB null) erythrocytes (Rowe et al., 1997).

Another possibility is that the protective mechanism is not directly via the GYPA molecule itself, but through its association with Band 3 to form the Wright^{b} antigen (Bruce et al., 1995). This hypothesis will be further developed throughout Chapters 4 and 5 of this thesis.

1.7.6 Glycophorin C (GYPC, CD236)

Glycophorin C (GYPC), responsible for the Gerbich blood group, is a known *P. falciparum* invasion receptor which binds Erythrocyte Binding Antigen 140 (EBA-140) expressed on merozoites (Maier et al., 2003, Mayer et al., 2006). Recently, two studies have suggested GYPC as a potential rosetting receptor for both *P. falciparum* and *P. vivax* (Lee et al., 2014, Niang et al., 2014). Lee *et al.* focussed mainly on *P. vivax* but also assessed the ability of BRIC 4 and BRIC 10 GYPC monoclonal antibody fragments to inhibit rosette formation in ten fresh *P. falciparum* clinical isolates from Thailand (Lee et al., 2014). They reported a significant decrease in rosetting with BRIC 4 but not BRIC 10

antibody fragments, though the reduction was only around 5-10% and counts were based on a single experiment with no replication (Lee et al., 2014). A GYPC knockdown was also produced using RNA interference methods in haematopoietic stem cells and the knockdown erythrocytes showed a more convincing reduction in rosetting, but only *P. vivax* isolates were assessed (Lee et al., 2014). Niang *et al.* used both soluble GYPC and a different GYPC antibody, Ret40f, to reduce rosetting in the laboratory line 5A-R+ (derived from 3D7) (Niang et al., 2014). They also produced GYPC knockdown erythrocytes and found minimal rosetting compared to wildtype cells, again with the single laboratory strain (Niang et al., 2014).

There are a number of naturally occurring altered GYPC phenotypes, though the true GYPC null, known as the Leach phenotype, is rare (Telen et al., 1991). It is due to the deletion of exons 3 and 4 of the GYPC gene resulting in hereditary elliptocytosis (Telen et al., 1991), and the rosetting ability of these cells has not been tested. More common, particularly in Melanesian populations (Patel et al., 2001), are the Yus and Gerbich phenotypes, arising from deletions in exons 2 and 3 respectively and resulting in a shortened GYPC molecule with altered glycosylation (Jaskiewicz et al., 2018). Gerbich negative variants (Ge-2,3, Ge2,-3, Ge-1, -2, -3) have been tested and reportedly rosette normally (Rowe et al., 1997, Goel et al., 2015).

The importance of GYPC as a rosetting receptor remains unclear, however there appears to be sufficient evidence to support further investigation with a wider range of parasites lines.

1.7.7 IgM and α2- macroglobulin

Rosetting parasites can be classified according to those which bind non-immune IgM and those which do not (Rowe et al., 2002c, Ghumra et al., 2012), with IgM positive binding being associated with malaria severity (Rowe et al., 2002c). The binding site on IgM has been identified as the Cμ4 domain of the Fc portion (Ghumra et al., 2008), with the corresponding PfEMP1 binding site within the DBLε and DBLζ domains (Semblat et al., 2006, Rasti et al., 2006, Ghumra et al., 2008, Stevenson et al., 2015a), i.e. some distance from the NTS-DBLα domain thought to be key for binding with host erythrocyte receptors. The minimal binding site for the DBL4ζ of the TM284var1 variant has been localized to subdomain 2 and parts of subdomains 1 and 3 (Semblat et al., 2015). While IgM itself is not sufficient, or always necessary, for rosetting to occur, it is hypothesized to strengthen rosettes and may act by cross-linking PfEMP1 molecules (Scholander et al., 1996, Clough et al., 1998a, Stevenson et al., 2015a).

More recently, α_2-macroglobulin has been proposed as an important serum rosetting factor for some parasites, which might also promote PfEMP1 cross-linking (Stevenson et al., 2015b). Stevenson *et al.* found that both α_2-macroglobulin and IgM bound the DBLζ2 domain from HB3var06 and the two molecules worked together synergistically in promoting rosette formation. Of note, α_2-macroglobulin was also sufficient to allow rosetting in serum-free media (Stevenson et al., 2015b). Of the 12 Ghanaian clinical isolates also tested, less than half bound IgM or α_2-macroglobulin (4 for α_2-macroglobulin and 5 for IgM) and no statistically significant correlation with severe disease was found, though the authors note the study was not designed or powered to test for any such correlation.

1.7.8 Unidentified receptors

None of the candidate receptors discussed above fully account for the rosetting interactions between infected and uninfected erythrocytes and many questions remain about the relative importance of each. Therefore it is likely that other, as yet unidentified, receptors are involved. There is evidence to suggest that these unknown receptor candidates are carbohydrates, as uninfected group O RBCs treated with trypsin, other proteases and Heparinase III are still able to rosette (Udomsangpetch et al., 1989, Rowe et al., 1994).

Table 1-2 Summary of host erythrocyte rosetting receptors. From (McQuaid & Rowe, 2019 submitted).

NAME	CHARACTERISTICS	STUDIES*	COMMENTS
ABO BLOOD GROUP ANTIGENS	Differ based on sugar at antigen terminus: A= N-acetyl-D-galactosamine, B= D-galactose, O= none O predominant blood group in Africa (Cserti and Dzik, 2007) Blood group O protects against severe malaria (Rowe et al., 2007, Tekeste and Petros, 2010, Malaria Network, 2014)	Parasites from patients with group O have reduced rosetting (Rowe et al., 1995, Rowe et al., 2007, Rout et al., 2012) Rosettes from group O patients are more easily disrupted by immune sera (Barragan et al., 2000b) *In vitro* rosetting rates higher for some parasites cultured in A, B, AB compared to O (Carlson and Wahlgren, 1992, Udomsangpetch et al., 1993, Barragan et al., 2000b) Removal of A/B antigen decreases rosette size (Barragan et al., 2000b) Blood group antigen binding site mapped to NTS-DBLα domain of PfEMP1-VarO (Vigan-Womas et al., 2012)	Rosetting does occur in group O Challenging to manipulate therapeutically Group A may be particularly relevant for RIFINs (Goel et al., 2015)
COMPLEMENT RECEPTOR 1 (CR1)	Membrane glycoprotein responsible for regulating the complement system Polymorphisms affect copy number, molecular weight and sequence	Rosetting reduced in CR1 deficient RBCs (Rowe et al., 1997) Soluble CR1 and CR1 antibodies disrupt rosettes (Rowe et al., 1997, Rowe et al., 2000, Lee et al., 2014)	Association with disease severity may depend on transmission intensity Soluble CR1 has been used therapeutically in humans in other fields (e.g. cardiac and renal disease (Lazar et al., 2004, Zhang et al., 2013))

		Essential region mapped to C3b binding site on LHR B and C (Rowe et al., 2000) Protection afforded by CR1 deficiency depends on transmission intensity and may be detrimental in areas of low transmission (Nagayasu et al., 2001, Cockburn et al., 2004, Teeranaipong et al., 2008, Sinha et al., 2009, Rout et al., 2011, Panda et al., 2012). *SI2* polymorphism reduces the risk of cerebral malaria and death, particularly in the absence of α-thalassaemia. Conversely, the McC^b phenotype increased the odds of cerebral malaria. (Opi et al., 2018)	
HEPARAN SULFATE (HS)**	Glycosaminoglycan Differs from heparin which has higher levels of sulfation and is only found in mast cells Invasion receptor for hepatocytes (Frevert et al., 1993) and red cells (Boyle et al., 2010)	Heparin disrupts rosettes in approximately half tested isolates (Udomsangpetch et al., 1989, Carlson et al., 1992, Rowe et al., 1994, Barragan et al., 1999) Heparinase treatment reduced rosetting in lab strains FCR3S1 and TM284(Barragan et al., 1999) Heparin binds to rosetting infected erythrocytes (Barragan et al., 2000a, Heddini et al., 2001)	Limited evidence that HS is present on mature RBCs (Vogt et al., 2004) Clinical trials of low anticoagulant heparin ongoing (Charunwatthana et al., 2014)

		Both heparin and HS bind the DBL-1 domain of FCR3S1.2 PfEMP1 (Barragan et al., 2000a)	
CHONDROITIN SULFATE (CS)	Glycosaminoglycan Binds specific PfEMP1 variant VAR2CSA in placental malaria (Desai et al., 2007)	Soluble CS did not disrupt rosettes (Rogerson et al., 1994, Rowe et al., 1994, Barragan et al., 1999) CS did not bind to DBL-1 domain of FCR3S1.2(Barragan et al., 2000a) Chondroitinase treatment reduced rosetting in one strain only (Barragan et al., 1999)	Minimal evidence of role in rosetting
CD36	Widely distributed membrane protein and scavenger receptor Deficiency fairly common in Africa (Fry et al., 2009)	Binds PfEMP1variants associated with mild disease (Kraemer and Smith, 2006, Kyriacou et al., 2006) Antibodies disrupt rosettes in single strain only (Handunnetti et al., 1992b)	Minimal evidence of role in rosetting Deficiency not associated with malaria severity (Fry et al., 2009)
GLYCOPHORIN C (GYPC)	Red cell membrane protein responsible for Gerbich blood group Red cell invasion receptor (Maier et al., 2003)	GYPC antibodies disrupted rosettes in a lab strain and Thai isolates (Lee et al., 2014, Niang et al., 2014) Reduced rosetting in GYPC knockdown RBCs (Niang et al., 2014) (single lab adapted parasite strain tested)	Possible role in *Plasmodium vivax* rosetting (Lee et al., 2014) Associated with STEVOR binding (Niang et al., 2014)
GLYCOPHORIN A (GYPA)	Sialoglycoprotein which, along with Glycophorin B, constitutes the MNS blood group	Red cells deficient in GYPA showed reduced rosetting with RIFIN transfected parasites (Goel et al., 2015)	Suggested to bind RIFINs in the absence of blood group A (Goel et al., 2015)

	Red cell invasion receptor (Bei et al., 2010)	Soluble sialic acid and neuraminidase or trypsin treatment reduced rosetting with RIFIN transfected parasites cultured in blood group O only (Goel et al., 2015) Soluble GYPA did not disrupt rosettes in strain 5A-R+ (Niang et al., 2014)	
UNKNOWN RECEPTOR/S	Possibly carbohydrate	Protease treated RBCs still capable of forming rosettes (Udomsangpetch et al., 1989, Rowe et al., 1994)	Mechanism could be accessory or independent

*Parasite strains used are not consistent between studies with a wide range of lab adapted and field isolates in use. Results are therefore not necessarily generalizable to all strains and within each study may apply to some tested parasites only.
**Many studies included here use heparin instead of/in addition to heparan sulfate.

1.8 Potential for anti-rosetting therapies

1.8.1 Disruption of rosettes

The diversity and complexity of potential rosetting mechanisms described above, from both the parasite and host perspective, presents a challenge for designing rosette disrupting therapies. However, this is an important avenue to explore as parasites can continue to exhibit cytoadherence after administration of anti-malarial drugs, potentially contributing to mortality and morbidity even after treatment (Hughes et al., 2010). *In vitro*, current anti-malarials including atovaquone, artesunate, chloroquine and quinine do not reduce rosetting, though screening has identified a Malaria Box compound, MMV006764, which showed some modest rosetting disrupting and anti-plasmodial capability (Ch'ng et al., 2016). Anti-PfEMP1 antibodies have been used *in vitro* as targeting molecules attached to the anti-malarial lumefantrine forming a 'drug-loaded immunoliposome' (Moles et al., 2016), which had both anti-rosetting and growth inhibition actions. However, the effects were specific to the parasite strain from which the antibodies had been derived, and more complex pools of antibodies have yet to be tested (Moles et al., 2016).

Heparin and other sulfated glycoconjugates such as curdlan sulfate and fucoidan are effective at disrupting rosettes (Rowe et al., 1994, Kyriacou et al., 2007) but as mentioned above, their anti-coagulant activity limit their therapeutic potential and heparin is especially not recommended for the treatment of severe malaria (WHO, 1986, WHO, 2015a). Chemically generated low anticoagulant forms of heparin have more potential (Skidmore et al., 2008) and disrupted rosettes by over 15% in the majority (89%) of fresh African clinical isolates (Leitgeb et al., 2011) and samples from Thai patients (Saiwaew et al., 2017) tested *in vitro*. *In vivo* sequestration in rats and macaque monkeys was also reduced (Vogt et al., 2006). Human phase I/II clinical trials of one such low anti-coagulant heparin, Sevuparin, have

taken place and some evidence of desequestration was seen with no obvious safety concerns (Leitgeb et al., 2017).

1.8.2 Anti-rosetting vaccines

As discussed above, different parasites appear to utilize different host receptors at different times, therefore it is likely that only a certain proportion of rosetting parasites would be susceptible to rosette disruption by targeting a single receptor. Prevention of rosette formation, possibly through adding an anti-rosetting component to the multi-component malaria vaccines currently in development, is therefore an attractive prospect. Antibodies from rabbits immunized with various PfEMP1 domains (NTS-DBL1α, DBLα, DBL3ε, DBL3δ, CIDR2β, NTS-DBLα-CIDR1γ) derived from the R29 parasite have also been shown to inhibit and disrupt rosettes and promote phagocytosis in a strain specific manner (Ghumra et al., 2011). The anti-NTS DBL1α antibodies was the most effective (Ghumra et al., 2011), and the DBL1 domain from the Palo Alto VarO strain showed the same effect (Guillotte et al., 2015, Guillotte et al., 2016). Correct folding of the PfEMP1 domain appears to be key and various expression systems have been successful in producing antigenic proteins (Guillotte et al., 2015). Further studies have shown that monoclonal antibodies generated from mice immunized with a NTS-DBL1α domain varied in their ability to either disrupt rosettes and/or promote phagocycytosis and these abilities were not necessarily correlated (Quintana et al., 2016). Promisingly, pools of polyclonal antibodies obtained through immunization of rabbits with the NTS-DBLα domains from different IgM-binding, rosetting parasites have been successful in inhibiting rosette formation and promoting phagocytosis in a diverse range of heterologous parasites strains, including African clinical isolates (Ghumra et al., 2012). In this study, there was a clear distinction between parasites which were IgM binding or not; antibodies to IgM binding parasites (HB3R+, TM284R+ and IT/PAR+) were able to bind to infected erythrocytes and disrupt rosettes of heterologous strains which were also IgM binding, but the same effect was

not seen for non-IgM binding parasites (Muz12R+, TM180R+ and IT/R29) (Ghumra et al., 2012). This is encouraging in terms of developing a useful anti-rosetting vaccine. Antibody responses have been seen in several different species (Ghumra et al., 2012, Angeletti et al., 2013, Guillotte et al., 2015) and further work is therefore required in determining which PfEMP1 domains derived from which strains are capable of inducing cross-reactive antibodies, and which adjuvants and dose schedules are most effective with a view to translating this work into humans.

1.9 Summary and remaining questions

The most convincing evidence that a host receptor is important for rosetting, is demonstrating that erythrocytes lacking the receptor of interest show reduced rosetting with infected erythrocytes across a wide range of diverse clinical and laboratory adapted parasite lines. From a therapeutic point of view, it is useful to show that rosettes can be disrupted by blocking, or otherwise interfering, with the receptor, without causing harm to the host. Unfortunately, none of the receptors described above fully meet these criteria. The evidence that blood group antigens, particularly group A, have an important role in rosetting is perhaps the most convincing, however designing a therapy targeting these receptors is extremely challenging; IgM atnibodies to blood group antigens can cause potentially fatalhaemolysis and such a therapy would not benefit those with blood group O. . Though naturally occurring low CR1 cells and GYPC knockouts have been tested, reduced rosetting was only seen in a subset of parasites, and the GYPA data is conflicting. Heparin does appear to be effective at disrupting rosettes across a broad range of clinical and laboratory parasites, though the evidence for HS as a specific rosetting receptor is incomplete. As such, a thorough investigation into the GAGs, HS and CS, forms a substantial part of this work.

There is therefore a pressing need to develop new methods for investigating and screening for rosetting receptors. The generation of multiple, well-characterized knockout erythrocytes would be an extremely useful tool for rosetting research. Such cells could be tested with multiple parasites lines and used for a large-scale screen such as that designed by Egan *et al.* (Egan et al., 2015). Until recently, the ability to generate usable quantities of genetically engineered knockout erythrocytes has been hampered by the lack of appropriate cell lines, but the advent of CRISPR-Cas9 technology (Doudna and Charpentier, 2014) and the development of immortalized erythroid lines (Kurita et al., 2013, Kanjee et al., 2017, Trakarnsanga et al., 2017) may allow these obstacles to be overcome. The aim of this thesis is therefore to reflect back by re-evaluating the evidence for existing receptors, specifically HS, and look forward by developing new methods and identifying novel, strain-transcending rosetting receptors using erythrocyte knockouts.

1.10 Aims of this thesis

The overall aim of this thesis is to identify the key receptors essential for rosetting in *P. falciparum* malaria, in order to inform the development of therapies specifically targeting severe forms of the disease. This will be achieved using three different lines of enquiry:

1. The ***investigation and re-evaluation*** of the role of the glycosaminoglycans, ***heparan sulfate and chondroitin sulfate***, as potential rosetting receptors:
    a. How does the specific removal of HS/CS affect rosetting?
    b. Are these molecules present on mature erythrocytes?

2. The use of ***cultured haematopoietic stem cell derived erythroid cells*** (cRBC) to investigate potential rosetting receptors and identify ***novel targets***:
    a. How does the cell surface receptor complement of cRBC from haematopoietic stem cells compare to mature peripheral erythrocytes?
    b. Do cRBC rosette?
    c. Can we use differences in rosetting between cRBC and peripheral erythrocytes to identify novel rosetting receptors?

3. Creating ***knockout erythrocytes*** using CRISPR-Cas9 technology and a ***novel immortalized erythroid line***:
    a. Are erythroid cells generated from immortalized lines suitable for use in rosetting research?
    b. Do knockout erythroid cells rosette?

2. CHAPTER II: MATERIALS AND METHODS

This chapter describes the procedures for *in vitro* culturing of *P. falciparum* parasites and the various other cell types used throughout this work, along with general protocols for frequently recurring techniques. Further details of the cell lines and specific methods are included at the beginning of each results chapter.

All culturing was undertaken in a containment level 3 (with derogations) laboratory within a Class II microbiological safety cabinet under sterile conditions.

2.1 Culturing *Plasmodium falciparum*

2.1.1 Parasite lines

A number of different culture-adapted *P. falciparum* lines have been used throughout this work in an, albeit limited, attempt to reflect the diversity of the parasite in the field. These have ranged from well-established and highly characterized lines such as ITvar60/Palo Alto, to those which have been more recently adapted to culture from the field such as 9605. The lines and their known characteristics are listed in Table 2-1. By necessity, these are all rosetting parasites, and it is important to acknowledge that they therefore represent a small proportion of an already select group. Field studies have shown that around 90% of parasites isolated from Malian children with severe malaria were able to form rosettes, compared to 55% from children with uncomplicated malaria, and the median rosette frequencies were 20% and 1% respectively (Doumbo et al., 2009).

Table 2-1 Parasite lines used in this thesis.

Parasite line (alternative names)	Presumed Origin and first publication	Known characteristics
ITvar60 (Palo Alto, PAR+, FCRS1)*	South East Asia (Robson et al., 1992, Mu et al., 2005) First publication- (Udomsangpetch et al., 1989)	Blood group A preferring (Barragan et al., 2000b)
R29/ITvar9*	South East Asia (Mu et al., 2005) First publication - (Roberts et al., 1992)	Highly dependent on CR1 for rosetting (Rowe et al., 1997)
9605	Kilifi, Kenya First publication - (Abdi et al., 2017)	Isolated from a child with cerebral malaria in 2009 (Abdi et al., 2017)
11019	Kilifi, Kenya Not yet published	Isolated from a child with cerebral malaria in 2009 (Personal communication P. Bull)
TM284R+	Thailand (Mu et al., 2005) First publication - (Scholander et al., 1996)	Blood group B preferring, rosettes sensitive to disruption by soluble chondroitin sulfate (Barragan et al., 2000b)
HB3R+	Honduras (Bhasin and Trager, 1984) First publication (of rosetting variant)-(Kyriacou et al., 2007)	The original HB3 did not rosette but has been selected for rosetting (see methods below and (Kyriacou et al., 2007))

**Of note, there is some uncertainty of the true origin of IT-derived parasite lines such as ITvar60 and ITvar9. Although the IT isolate originally came from Brazil, the culture-adapted parasite line is thought to have been cross-contaminated with another line some 30-40 years ago (Robson et al., 1992). PAR+ and R29 are clones derived from the strain IT which express different PfEMP1 variants (Rowe et al., 1997) Molecular typing indicates that IT-derived parasites have a South East Asian origin (Mu et al, 2005).*

2.1.2 *P. falciparum* culture methods

Parasites were cultured in RPMI-1640 media (Lonza, BE12-167F) supplemented with 2mM L-glutamine (Invitrogen,. 25030-024), 25mM Hepes (Lonza, 17-737F), 16mM Glucose (Sigma, G8270), 25µg/ml Gentamicin (Lonza 17-518Z), and 1M sodium hydroxide (Fisher Scientific, S/4920/60) added to give pH 7.2-7.4. This 'incomplete media' was supplemented with 0.25% AlbuMAX^{TM} II Lipid-Rich BSA (Gibco, 11021-037) and 5% pooled human serum (Scottish National Blood Transfusion Service). Parasites were cultured in non-vented flasks (ranging from 25-150cm^2 depending on volume of media required) which were gassed for 30-60 seconds (depending on flask size) with a mixture of 1% oxygen, 3% CO2 and 96% Nitrogen (BOC Limited, 228446-L) and incubated at 37^{o}C.

The parasite media was changed daily, except over the weekend. A 250µl sample was taken from the culture flask each day and used to determine the parasitaemia (as detailed below). The entire culture was then centrifuged at 900g for 4-5 minutes and the supernatant aspirated. Fresh media was added to give a final haematocrit of 2% (1% over the weekend) and the parasitaemia was adjusted to the required percentage (for maintenance cultures, approximately 2-5%; for rosetting experiments, 5-10%) by adding human erythrocytes prepared and stored as described in section 2.1.3.

2.1.3 Processing donor blood for culturing

In general, parasites were cultured in blood group O+, unless otherwise stated. Blood for parasite culturing was obtained from volunteer Scottish donors via the Scottish National Blood Transfusion Service based in Edinburgh, UK (ethical review reference number 15-37). White blood cells were removed by passing the contents of the blood pack through the leukodepletion filter provided with the pack with the aid of gravity, and the filtered red cells aliquoted into 15 or 50ml Falcon tubes. These tubes were then centrifuged at 2400g for 5 minutes and the plasma removed.

Erythrocytes were then washed twice with 10ml of incomplete RPMI by spinning at 2400g for 5 minutes, removing the supernatant then repeating. An equal volume of incomplete RPMI was then added to the remaining packed cells to give a 50% haematocrit and the blood was stored at 4°C. Blood was used for a maximum of two weeks after washing.

2.1.4 Determination of parasitaemia using Giemsa staining

A 250µl sample of the parasite culture was taken and the red cells pelleted by brief centrifugation. The majority of the supernatant was removed, leaving sufficient to resuspend the pellet at approximately 40% haematocrit. A thin smear was made onto a glass slide which was then dried, fixed with Methanol, dried again, then stained with filtered 10% Giemsa solution (TCS Biosciences, HS295-506 in Giemsa buffer, Merck, TP936968 805) for 25 minutes. The stain was rinsed off with water and the smear visualized with a 100x oil immersion objective using an Olympus BX45 light microscope. Five hundred cells from several different fields were counted and scored as uninfected, infected with ring stage parasite or infected with mature parasite (pigmented trophozoite or schizont) and the percentage parasitaemia calculated.

2.1.5 Selection for rosetting

Parasites were selected for rosetting using two different, alternating methods; Percoll (Aley et al., 1984, Handunetti et al., 1990) or flotation in 3% Gelatin/0.9% Saline (Gelofusine) (Handunnetti et al., 1992a). For both methods, selection was performed when the majority of the parasites were at the mature pigmented trophozoite stage.

2.1.5.1 Percoll selection

A solution of 60-70% Percoll was made by first adding 10ml of 10x RPMI (Sigma, R1145) to 90ml of Percoll (GE Healthcare, 17-0891-01), to give 90% Percoll, then taking 33.3ml of the 90% solution and adding 16.7ml of incomplete RPMI to give 60% Percoll (volumes were adjusted to give different percentage solutions as required). The parasite culture was pelleted then resuspended in 5ml of complete RPMI and gently layered over 7ml of the Percoll solution in a 15ml tube using a serological pipette. The tube was then centrifuged at 2400g for 10 minutes. This resulted in an upper band of non-rosetting trophozoites at the Percoll/RPMI interface and a pellet of the remaining cells; uninfected and rosetting trophozoite/ring infected red cells. The supernatant and non-rosetting band were discarded and the remaining pellet washed twice with incomplete RPMI then placed back in culture.

2.1.5.2 Gelofusine selection

The parasite culture was centrifuged and resuspended at 50% haematocrit with incomplete RPMI in a 15ml Falcon tube. An equal volume of Gelofusine (Braun PL, 03551/0047) was added and the tube incubated, upright, at 37ºC for 15 minutes. This resulted in the formation of two separate layers, the top contained the non-rosetting knob-positive trophozoites and the bottom the remaining cells. The top layer was removed and the bottom washed once in incomplete RPMI and once in complete RPMI before being placed back in culture.

2.1.6 Synchronisation of parasites

Parasites were kept synchronised at the same lifecycle stage using 5% D-Sorbitol (5g of Sorbitol, [Sigma, S-3889] in 100ml of dH_2O, sterilized by autoclave) which lyses mature parasites which are more permeable to Sorbitol (Lambros and Vanderberg, 1979). The culture was pelleted and resuspended in 10ml of 5% Sorbitol and incubated for 15 minutes at 37ºC.

The solution, now containing only ring stage parasites, was then washed twice with incomplete RMPI and placed back in culture with complete RPMI.

2.1.7 Freezing and thawing of parasites

Parasites were thawed by placing a frozen vial in the water bath until the contents had just melted. The suspension was transferred to a 50ml Falcon tube and 200µl of 12% sodium chloride added (12g of NaCl [BDH, 10241AP] in 100ml dH_2O, sterilized by autoclave) very slowly by droplet with continuous mixing. The suspension was left to stand for 5 minutes, then 10ml of 1.8% sodium chloride (1.8g of NaCl [BDH, 10241AP] in 100ml dH_2O, sterilized by autoclave) was added in the same manner, followed by 10ml of 0.9% sodium chloride with 0.2% glucose (0.9g of NaCl, BDH, 10241AP, and 0.2g glucose, Sigma, G8270, in 100ml dH_2O, sterilized by autoclave). The suspension was centrifuged for 4 minutes then washed with 20ml of incomplete RPMI before being placed into culture with a few drops of fresh blood.

The parasites were frozen by pelleting the culture in a 50ml Falcon tube, removing the supernatant then adding filter-sterilized Glycerolyte (made of 57g glycerol, 1.6g sodium lactate, 30mg potassium chloride, 1.38g sodium dihydrogen phosphate in 100ml of dH_2O) in a 2 stage- process. For the first stage, a volume of Glycerolyte equal to one third the pellet volume was added slowly and dropwise with constant mixing. The tube was left for 5 minutes then four times the original volume of Glycerolyte was added in the same way (giving a total of 5 volumes Glycerolyte to 3 volumes packed cell volume). One ml aliquots were placed in 1.8ml Nunc® CryoTubes® (Sigma, V7634) and transferred to a minus 70^{o}C freezer or liquid nitrogen for long term storage.

2.1.8 Magnetic activated cell sorting (MACS)

Infected erythrocytes were purified using Magnetic Activated Cell Sorting (MACS). This method relies on the haemozoin within the mature stage

parasites adhering to a strong magnet, while allowing the uninfected cells and ring-stage parasite to pass through (Miltenyi et al., 1990, Staalsoe et al., 1999). A VarioMACS^{TM} Separator (Miltenyi Biotec, 130-090-282) was used with CS columns (Miltenyi Biotec, 120-000-490). The column was fitted with a three way tap and 20G needle then equilibrated by running through 10ml of PBS, 15ml of incomplete RPMI then 5ml of incomplete RPMI with 0.5% BSA (Sigma, A8577-1L) and 0.1-1mg/ml heparin (Sigma, H4784, concentration dependent on parasite heparin sensitivity). In parallel, the parasites were prepared by washing a culture aliquot contained 200µl of packed cell volume with incomplete RPMI then resuspending in 10ml of incomplete RPMI/0.5% BSA and heparin in order to disrupt the rosettes. The concentration of heparin required varied with the parasite strain but ranged from 0.1-1mg/ml. The heparin/erythrocyte mixture was incubated at room temperature for 5 minutes then a 10µl sample checked by microscopy to ensure all rosettes had been disrupted. Once the rosettes had been fully disrupted, the parasite solution was added to the top of the MACS column and allowed to run through with the flow rate controlled using the 3-way tap to approximately 1 drop per second. The flow-through was collected and added back to the top of column and allowed to passed through the column a second time. Once the parasite solution has passed through a second time, a smear slide of the flow-through was made to confirm all infected erythrocytes had been removed, and the column was washed with 10-20ml of incomplete RPMI with heparin and 0.5% BSA. The column was then removed from the magnet and 10ml of incomplete RPMI/0.5% BSA/heparin plunged into the column using a 20ml syringe through the side port of the three way tap. The liquid was withdrawn then plunged back in and out of the column several times to obtain the infected erythrocytes which had adhered to the magnet, before being place in a 15ml tube. A 500µl aliquot of the parasite containing solution was taken and a thin smear made to check the percentage purity of the parasites. The remainder of the cells in the heparin solution were centrifuged for 4 minutes at 900g and the supernatant aspirated. The pellet was resuspended in incomplete RPMI without heparin and centrifuged again to wash the cells

two further times. After washing, the purified parasites were resuspended in complete RPMI and used immediately for experiments. If multiple purified parasite lines were required on the same day, the smaller QuadroMACS™ cell separator was used along with the corresponding LS columns (Mitenyi Biotec,130-042-401). The columns and parasites were prepared in the same manner, however only 50µl of PCV was purified and the parasite solution allowed to flow through by gravity, not controlled by a 3-way tap.

2.2 Culturing and maturation of haematopoietic stem cells (HSC)

Frozen, human, adult bone marrow CD34+ cells were purchased from Stemcell™ technologies (www.stemcell.com, 70002). All donors (with one exception, see Table 2-2) were of Caucasian origin to minimise the possibility of the cells containing malaria-protective polymorphisms that could influence rosetting, and all had tested negative for HIV, Hepatitis B and C. Additional frozen, human, adult bone marrow CD34+ cells were also purchased from Lonza (Lonza Poietics™, 2M-101). The characteristics of each of the donors provided by the companies are listed in Table 2-2. Two vials of human umbilical cord haematopoietic stem cells were a kind gift from Professor Lesley Forrester (Centre for Regenerative Medicine, University of Edinburgh).

Table 2-2 Characteristics of CD34+ haematopoietic stem cell donors.

Company	Donor number	Age (yrs) / Gender	Ethnicity	Height/weight (cm/kg)	Blood group
SCT	D001004313	40/F	Caucasian	156/57	UNK
SCT	D001003739	37/F	Caucasian	176/75	UNK
SCT	1002328	55/M	Caucasian	176/98	A+
SCT	1001448	46/M	Caucasian	182/92	A+
SCT	D001004150	30/M	Caucasian	181/84	B+
SCT	D001003739	37/F	Caucasian	176/75	O+
SCT	1000779	49/M	Caucasian	180/94	O+
Lonza	0000578848	29/F	Caucasian	UNK	UNK
Lonza	0000521847	28/F	Caucasian	UNK	UNK**
Lonza	0000573127*	26/M	Asian	UNK	UNK

**This donor was selected and shipped by Lonza in error. Cells were used in preliminary culturing experiments only and not in rosetting assays. **Did not express A antigen on flow cytometry.* SCT= Stem cell technology, F= female, M= male, UNK= information not available.

2.2.1 Thawing of CD34+ HSC

A frozen vial of HSC was placed in a water bath at 37^{o}C until just thawed, then transferred to a 15ml Falcon tube to which 9ml of pre-warmed culture medium (see below) was added, dropwise with gentle mixing. The cells were then centrifuged at 300g for 10 minutes, the supernatant aspirated and the pellet resuspended at the appropriate density for the age of the cells as described below (i.e. 1x10^4 cells/ml for a fresh vial or 6x10^4 cells/ml for cells frozen on day 6 of culture) before being placed in 25cm^2 vented flasks.

2.2.2 CD34+ HSC cell culture methods

The CD34+ haematopoietic stem cells, hereafter referred to as cRBC, were cultured using a three-stage technique adapted from (Giarratana et al.,

2011). The protocol used here was kindly provided by Dr Jo Mountford (University of Glasgow). Cells were thawed as described above, and resuspended in base media consisting of Iscoves basal media with 4mM Glutamine (Biochrom AG, FG0465M), 5% Human AB serum, ("off the clot" and unfiltered, TCS Biosciences, CR300-500), 3U/ml heparin (Sigma, H4784), 10µg/ml Human Insulin solution (Sigma, I9278) and 200µg/ml Holo-Transferrin (Sigma, T0665). The base media was supplemented with day 0-8 additives (Table 2-3) and the cells placed in a 25cm^2 vented flask which was kept in a 5% CO2/air incubator at 37^{o}C. Table 2-3 illustrates the subsequent daily culturing process.

Of note, until day 8 the culture media was not fully changed, fresh media was simply added to give the required cell density as shown in Table 2-3. However, the additives were fully re-supplemented, for example, if 4ml of media was added to an existing 10ml already in the flask on day 6 then 14ml worth of additives were also added. On and after day 8 (except on day 15) the entire culture was centrifuged at 300g, supernatant removed and fresh media added with additives to give the required cell density. Cells were generally used for experiments up to day 21, as significant deterioration and cell death was noted after this point.

Table 2-3 Culturing protocol for cRBC

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 11	Day 15	Day 18	Day 21
Cell density (cells/ml)	$1x10^4$	$2x10^4$	$4x10^4$	$6x10^4$	$1\text{-}3x10^5$	$5\text{-}10x10^5$	$1\text{-}10x10^6$	$1\text{-}10x10^6$	$1\text{-}10x10^6$
Action	Thaw	Add media as required Add D0-8 additives	Add media as required Add D0-8 additives	Add media as required Add D0-8 additives	Centrifuge 300g, 10mins, resuspend in fresh media Add D8-11 additives	Centrifuge 300g, 10 mins, resuspend in fresh media Add D11-25 additives	Add media as required Add D11-25 additives	Centrifuge 300g, 10 mins, resuspend in fresh media Add D11-25 additives	Centrifuge 300g, 10 mins, resuspend in fresh media Add D11-25 additives
Additives	**D0-8**				**D8-11**	**D11-25**			
	SCF 60ng/ml (Peprotech, 300-07) IL3 5ng/ml (Peprotech, 200-03-2) EPO 3U/ml (Biolegend, 587102) Hydrocortisone 1μM (Sigma, H0888)				SCF 10ng/ml EPO 3U/ml Hydrocortisone 1μM Holo-Transferrin 300μg/ml (Sigma, T0665)	EPO 3U/ml Holo-Transferrin 300μg/ml			

2.2.3 Determination of cell count

Cells were counted using an improved Neubauer haemocytometer (depth 0.1mm, AC1000, Hawksley). Ten μl of well-mixed cell suspension was placed under the haemocytometer coverslip and two separate counts from different large squares on the haemocytometer grid were made then averaged. This number gave the number of cells $x10^4$ per ml and could therefore be used to calculate the total number of cells.

2.2.4 Filtration to remove nucleated cells

In order to obtain a pure, enucleated culture, cells could be filtered to remove those which still contained a nucleus. The number of cells in the culture were counted, then the required volume was washed once in incomplete RPMI then resuspended in 5ml (to maximum $1x10^8$ cells in 5ml) of warm incomplete RPMI. The plunger of a 10ml syringe was removed and a filter unit (Acrodisc Pall, 32mm syringe filter with 5μm Supor® membrane, 4650) attached. The syringe-filter set up was primed by running through 5ml of warm PBS with 2% BSA (PBS with 333μl of 30% BSA) after which the 5ml cell suspension was allowed to flow through into a 50ml Falcon tube using gravity. A further 5ml of incomplete RPMI was added to the top of the syringe and allowed to flow through. After this, a final 5ml of incomplete RPMI was added and the plunger reattached and slowly depressed. The resulting suspension was centrifuged for 15 minutes at 200g and the pellet washed once with incomplete RPMI before being resuspended as required.

Of note, even with a highly enucleated culture, the filtration process reduced the number of cells by 80-90% therefore in general, unfiltered cells were used in experiments. These unfiltered cells generally contained 70-90% enucleated cells.

2.2.5 Freezing cRBC

Cells were frozen up to and including day 7 of culture but not beyond. The cells were counted then centrifuged down at 300g for 5 minutes. The pellet was then resuspended in culture medium at $2x10^6$ cells/ml before adding the same volume of freezing medium (60% KnockOut™ serum replacement [Thermo Fisher A3181501], 20% DMSO and 20% culture medium). One ml aliquots were placed in 1.8ml Nunc® CryoTubes® (Sigma, V7634) and transferred to a minus 70°C freezer or liquid nitrogen for long term storage.

2.3 Culturing and differentiation of an immortalized erythroid line (EJ cells)

The immortalized erythroid line known as EJ cells was developed by Dr Erik J Scully, Dr Estela Shabani and others at Professor Manoj Duraisingh's laboratory at the Harvard T.H. Chan School of Public Health in Boston, USA. The cells used in this thesis were a kind gift from Professor Duraisingh and the following protocols are adapted from those developed by Dr Erik J. Scully and Dr Estela Shabani. Further details about the EJ cells are contained in the introduction of Chapter 4. Of note, all the EJ cells used, including wildtype EJ cells, have been transduced with a LentiCas9-Blast as the first stage of the LentiGuide-Puro two vector CRISPR-Cas9 protocol (Sanjana et al., 2014). The EJ cells therefore have a Blasticidin inducible Cas9 integrated into the cell line.

2.3.1 Thawing EJ cells

EJ cells were thawed by placing the frozen vial in a water bath at 37°C for 3-4 minutes then adding 10ml of pre-warmed pIMDM (see below), dropwise with gentle mixing in a 15ml Falcon tube. The cells were then centrifuged at 200g for 7-8 minutes, washed once in pIMDM then seeded at $5x10^4$ cells/ml in maintenance media.

2.3.2 EJ cell culture methods

2.3.2.1 Maintenance

EJ cells were maintained in their replicating and relatively undifferentiated state in maintenance media (referred to hereafter as pIMDM, Table 2-4), with the addition of 5% human AB serum, Dexamethasone, Doxycycline, Erythropoietin and Stem Cell Factor (for details and concentrations, see Table 2-5). The pH of the media was adjusted to approximately 7.4.

Table 2-4 Components of EJ base medium, pIMDM

	Final concentration (after serum added)
Iscove's liquid medium with stable Glutamine (AG Biochrom FG0465M)	4mM Glutamine
Holo-Transferrin (Sigma T0665)	330µg/ml
Human Insulin solution (Sigma I9278)	10µg/ml
Heparin sodium salt (Sigma H4784)	2IU/ml
Penicillin/Streptomycin 10,000U/ml (Gibco 15140122)	0.5% v/v

Both Dexamethasone and Doxycycline prevent differentiation further along the erythroid line and maintain the immortalization of the cells. Blasticidin was used to select for expression of Cas9 enzyme, and Puromycin to select for the knockout cells (Table 2-5), as required, however these reagents were not routinely added for daily culturing.

Table 2-5 Additives for complete EJ medium for maintenance, early differentiation and late differentiation stages.

	Maintenance media (final concentration)	**Early differentiation media** (final concentration)	**Late differentiation media** (final concentration)
Human AB Serum (TCS biosciences CR300-500)	5%	5%	5%
Dexamethasone (Sigma D4902)	1µM	Nil	Nil
Doxycycline hyclate (Sigma D9891)	1µg/ml	0.5µg/ml	Nil
Erythropoietin (Biolegend 587102)	3U/ml	3U/ml	3U/ml
Stem cell factor (Peprotech 300-07)	50ng/ml	10ng/ml	Nil
***Blasticidin S, hydrochloride** **(Calbiochem 203350)**	10µg/ml- when selection required	10µg/ml	10µg/ml
****Puromycin dihydrochloride** **(Calbiochem 540411)**	2µg/ml- when selection required	2µg/ml	2µg/ml

*For cells integrated with Cas9 **For Knockout cells only

Cells were seeded at $5x10^4$ cells/ml in vented $75cm^2$ flasks and grown in 5% CO_2 at 37°C. The medium was changed every 2-3 days by centrifuging the contents of the flask for 7-8 minutes at 200g, aspirating the supernatant and reseeding at $5x10^4$ cells/ml.

2.3.2.2 Differentiation

The immature, undifferentiated EJ cells were driven down the pathway towards maturity by modifying the additives to the base pIMDM and supporting the cells with a murine stromal line, MS-5 (Itoh et al 1989) as illustrated in Figure 2-1 and described below.

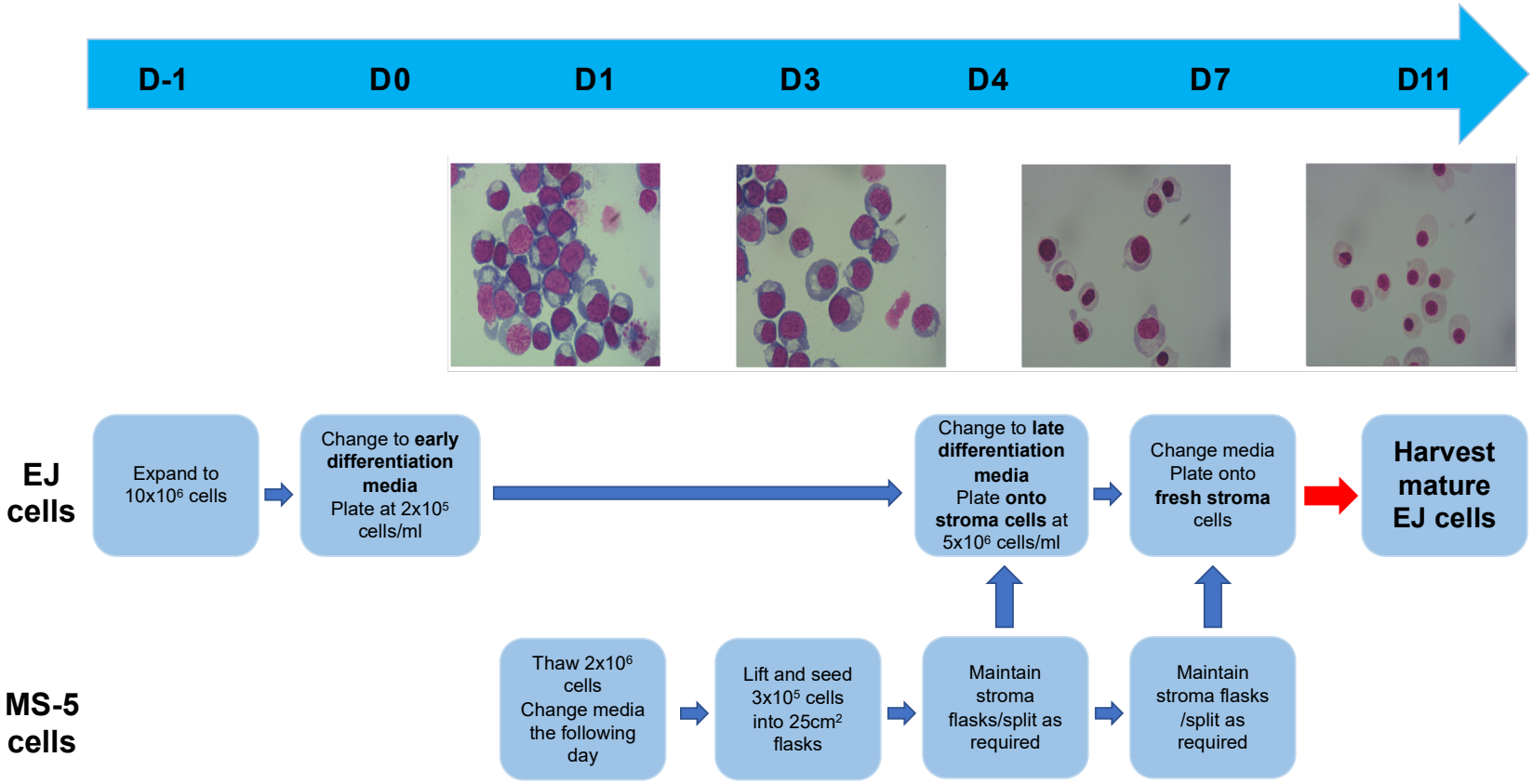


Figure 2-1 Differentiation of EJ cells to maturity supported by MS-5 cells. Images show cell morphology at using a x40 objective on an Olympus BX45 light microscope on day 0, 4, 7 and 11

Over the week prior to starting differentiation, the EJ cells were expanded to approximately 5-10x10^6 cells. According to Dr Shabani (personal communication), a further two- to four-fold expansion can be expected with a variable enucleation rate of 1-8%. However, I found that after an initial expansion, cell numbers reduced markedly after around 8 days due to cell death. On day zero, the EJ cells were centrifuged at 200g for 7-8 minutes then resuspended in pIMDM with early differentiation media additives (Table 2-5). The day after the start of the differentiation process (D1), 2 x10^6 MS-5 cells were thawed as described in section 2.5.2 and the media changed the following day. On day 3, the stroma cells were lifted and 3x10^5 cells per flask were seeded into 25cm^2 vented flasks in preparation for plating of the EJ cells. On day 4 the EJ cells were centrifuged at 200g for ten minutes then counted and resuspended at 5x10^6 cell/ml in pIMDM with late differentiation media additives (Table 2-5). The cell-containing media was pipetted onto MS-5 stromal cells (after aspiration of stroma media) and the flasks incubated at 37^{o}C in an incubator with 5% CO_2 as before. Of note the minimum and maximum volumes of EJ cells per 25cm^2 flask were 8 and 12ml, therefore if the resulting volume of media was less than 8ml, the cells were diluted further and if greater than 12ml then more than one stromal flask was used. On day 7, the EJ cell media was changed as described for day 4 and the cells replated on fresh stroma. Mature EJ cells were harvested on day 8-11 by gently aspirating the EJ cell suspension off the adherent stroma, centrifuging at 200g for 10 minutes then resuspending in pIMDM with late differentiation media. Cells could be stored at 4^{o}C for 1-2 days if required, though significant number of cells were lost during this brief period of storage.

2.3.3 Determination of cell count

Cells were counted as described in section 2.2.3.

2.3.4 Selection for undifferentiated cells using Percoll gradient

If the undifferentiated EJ cell pellet was noted to be turning pink/red indicating the beginnings of maturation, the cells were separated into early and late stages using 40% Percoll (Kanjee et al., 2017). Briefly, the cells were pelleted then resuspended in 4ml of pIMDM with 0.1% BSA then gently layered onto 4ml of 40% Percoll (GE Healthcare, 17-0891-01, see section 2.1.5.1) in a 15ml Falcon tube. The tube was then centrifuged at 1000g for ten minutes with low acceleration and deceleration. This resulted in the more mature cells pelleting at the bottom of the tube and the earlier, undifferentiated cells at the Percoll/medium interface (Figure 2-2). The separated cells were then washed three times in pIMDM and resuspended or discarded as required in maintenance media.

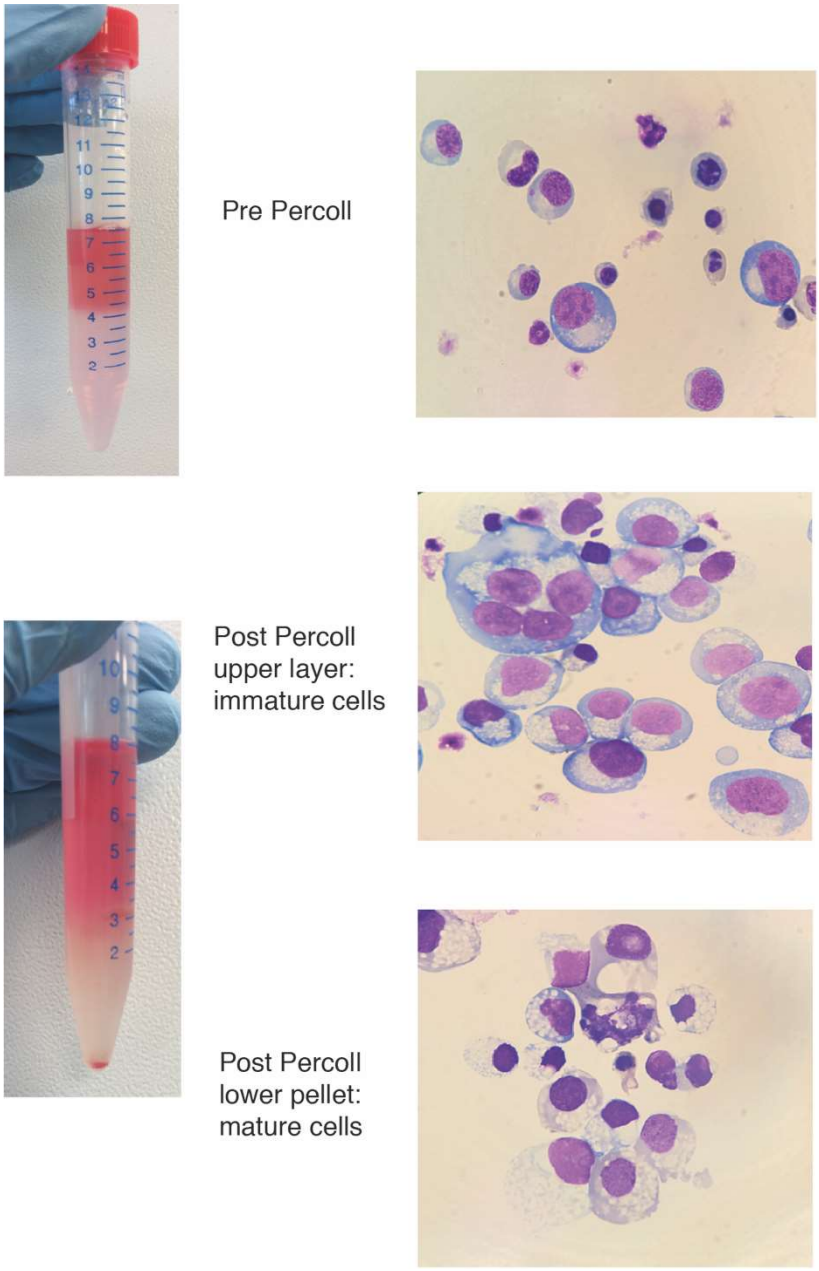


Figure 2-2 Separating immature/maturing EJ cells using 40% Percoll. Cells were stained with May-Grunwald/Giemsa as described in the text and visualized on an Olympus BX45 light microcope. Images were taken with an iPhone 5 camera phone.

2.3.5 Freezing of cells

The cells were counted then the required volume centrifuged for 7-8 minutes at 200g resuspended in 5ml of cold, sterile, heat-inactivated fetal bovine serum (FBS, Gibco, 10500-064) then centrifuged again at the same speed

for the same time. The cell pellet was then resuspended in sufficient FBS to given $2x10^6$ cell/ml and an equal volume of cold freezing solution (80% FBS and 20% DMSO) was added, slowly and dropwise with gentle mixing. One ml aliquots were placed in 1.8ml Nunc® CryoTubes® (Sigma, V7634) and transferred to a minus 70ºC freezer or liquid nitrogen for long term storage.

2.4 Culturing of COS 7 cells

2.4.1 Cell culture methods and passage

COS 7 cells were obtained from ATCC (https://www.lgcstandards-atcc.org/, ATCC® CRL-1651™). COS 7 cells were cultured in Dulbecco Modified Eagle Medium (Sigma, D6421) with 100U/ml Penicillin/0.1mg/ml Streptomycin (Gibco 15140122), 2mM L-Glutamine (Gibco, 25030-024), 10mM Hepes (Lonza, 17-737F) and 10% heat inactivated fetal bovine serum (Gibco 10500-064). Approximately 20-30ml of media was used per $75cm^2$ flask and cells were incubated at 37ºC in an incubator with 5% CO_2/air.

When the cells reached 80-100% confluency (usually every 3-4 days), flasks were split at a ratio of 1:4 or 1:5. The spent medium was removed by aspiration and the flasks washed twice with 10ml of sterile, warmed PBS. Three ml of Trypsin/EDTA (0.25%, Gibco, 25200056) was added and incubated for six minutes at 37ºC. The flasks were inspected on an inverted microscope to ensure the cells had lifted, then 10ml of culture medium with FBS was added and the whole suspension centrifuged in a Falcon tube for 10 minutes at 600g. The supernatant was removed, and the cell pellet resuspended in fresh medium and split as required.

2.4.2 Freezing and thawing of cells

A frozen vial of COS 7 cells was placed in a water bath at 37ºC until the contents had thawed, then 10 ml of culture medium was added and

centrifuged at 600g for 4 minutes. The supernatant was removed, cells resuspended in 10ml of fresh medium then placed in a 25cm_2 flask which was incubated 37^oC in 5% CO_2/air.

For freezing, cells were lifted as described above, washed once in culture medium then counted and resuspend at 1x10^6 cells/ml in freezing solution (90% culture medium with 10% DMSO) before transfer to a minus 70^oC freezer or liquid nitrogen.

2.5 Culturing of MS-5 stromal cells

2.5.1 Cell culture methods and passage

MS-5 murine stromal cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Drexler et al., 2001). Cells were cultured in 75cm^2 vented flasks in 5% CO_2 at 37^oC in Minimum Essential Medium α with nucleosides and GlutaMAX^{TM} (Gibco, 32571028), supplemented with 10% heat inactivated fetal bovine serum (Gibco 10500-064) and 0.5% v/v Penicillin/Streptomycin (Gibco 15140122).

Confluent cultures were split 1:3 every 2-3 days using the same method described in 2.4.1, except that the centrifuge speed was adjusted to 200g. This process constituted one passage. As the ability of MS-5 cells to support EJ cell differentiation is related to the passage number of the MS-5 cells (Estela Shabani, personal communication), the stromal cells were not used beyond passage 14.

2.5.2 Freezing and thawing of MS-5 cells

The MS-5 cells were thawed by placing the frozen vial in a water bath at 37^oC for 2-4 minutes then adding 10ml of pre-warmed culture medium, slowing and dropwise with gentle mixing. The suspension was then

centrifuged for 5 minutes at 200g, the supernatant aspirated and the cells seeded at 1-2x10^6 cells per 75cm^2 flask with 20ml of media per flask.

Cells were frozen by lifting the cells as described above, counting, centrifuging at 200g for ten minutes, and resuspending at 2x10^6 cells/ml in freezing medium (70% culture medium, 20% FBS and 10% DMSO). One ml aliquots were placed in 1.8ml Nunc® CryoTubes® (Sigma, V7634) and transferred to a minus 70°C freezer or liquid nitrogen for long term storage.

2.6 Rosetting assays

2.6.1 General determination of rosette frequency

The rosette frequency of parasites in culture was determined by taking a 250-500µl aliquot of culture into a 500µl Eppendorf tube and staining with 25µg/ml of ethidium bromide (1% stock, Sigma, 46067) for two minutes at 37°C. A 10µl aliquot of the stained culture was then place on a microscope slide and covered with a 22x22mm coverslip. The cells were viewed under combined florescence and white light using a Leica DM LB2 florescent microscope. Trophozoite infected erythrocytes were identified by the bright orange dot of fluorescence and two hundred of these infected cells counted and scored as rosetting or non-rosetting, based on whether or not they were adherent to at least two uninfected erythrocytes. The rosetting frequency was expressed as the percentage of infected erythrocytes forming part of a rosette compared to all infected erythrocytes. An example image from a rosetting culture prepared as above is shown in Figure 2-3.

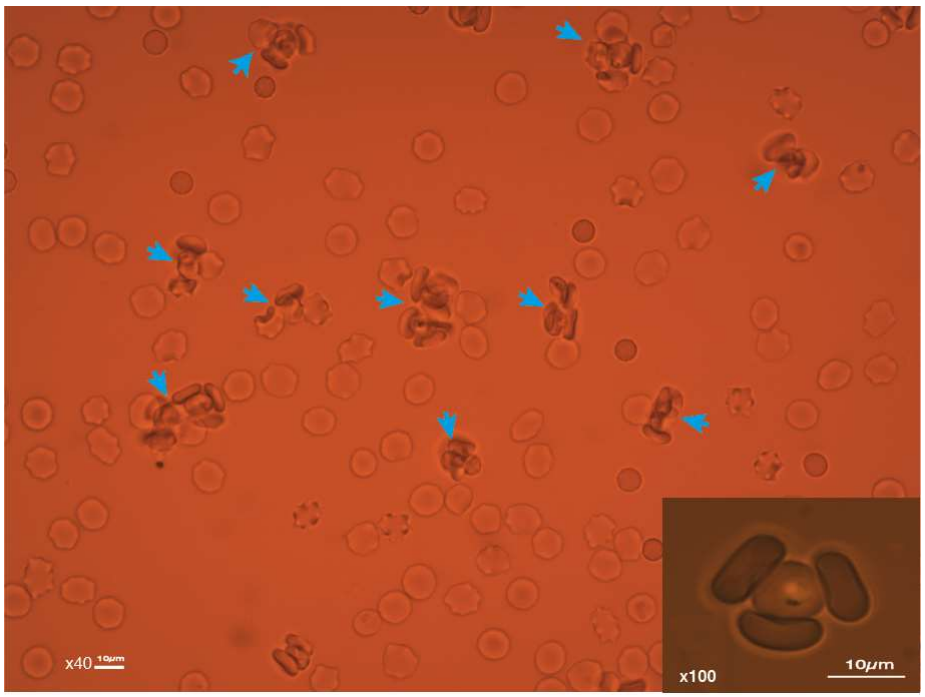


Figure 2-3 Rosetting parasite culture at x40 and x100 (inset) magnification. Rosettes are indicated with blue arrows.

2.6.2 Rosette disruption

A range of molecules, including heparin, antibodies, antibody fragments and soluble proteins were tested for their ability to disrupt rosettes. Further details are included in the relevant chapters, however the basic protocol for these rosette disruption experiments is described here. For rosette disruption assays, parasite infected cultures were washed and resuspended in complete RPMI binding medium which does not contain sodium bicarbonate, and therefore has a more stable pH than regular culture medium when carrying out experiments in which the parasites are not gassed. Binding medium consists of RPMI powder (Gibco 13018-015, contains Glutamine and HEPES) dissolved in distilled water with pH adjusted to 7.4, 16mM Glucose (Sigma, G8270), 25µg/ml Gentamicin (Lonza, 17-518Z), and 10% heat-inactivated human serum. Incomplete RPMI binding medium is complete binding medium as described above but without the addition of serum.

As rosetting only occurs at the pigmented trophozoite and schizont stage, synchronized cultures containing predominantly these mature stages were used for experiments. Generally, 2ml of mature-stage culture suspension at 2% haematocrit was pipetted into a 15ml Falcon tube and 2µl of ethidium bromide (i.e. 1:1000 of 1% ethidium bromide, Sigma 46067) added. This was incubated for 2 minutes at 37^{o}C then centrifuged for 4 minutes at 900g and the supernatant removed for disposal through charcoal-filtering and incineration as per protocol for ethidium bromide containing waste. The pellet was resuspended in 2ml of pre-warmed complete RPMI binding medium and 45µl aliquots placed into 1.5ml Eppendorf tubes. Five µl of the substances under investigation at the appropriate concentration were added, along with a positive control (usually Fucoidan, final concentration 100µg/ml, Molekula, 9072-19-9) and negative control (PBS) and all the tubes were blinded by a colleague before being incubated at 37^{o}C for 60 minutes with resuspension via gentle flicking every ten minutes. Once the incubation time was complete, 1.8µl samples were pipetted onto a multispot slide (12 well, 6mm, Hendley-Essex, PH001) and two hundred infected erythrocytes were counted and scored to give rosette frequency as a percentage. This was then repeated with a fresh sample on a second slide and the mean rosette frequency from the two counts (i.e. two technical replicates) was calculated for each sample.

2.6.3 Rosetting with cRBC and EJ cells

Rosetting assays with cRBC and EJ cells (Figure 2-4) were carried out by first MACS purifying the infected erythrocytes as detailed in section 2.1.8. The resulting pellet contained both infected erythrocytes, and a percentage of uninfected erythrocytes (usually around 20-30%, but potentially up to 50% of the cells were uninfected). In order to distinguish between these ‘contaminating’ erythrocytes, and the enucleated cRBCs/EJ cells, either the cRBC/EJs or the MACS purification pellet was stained with CellTrace^{TM} Oregon Green^{TM} 488. To stain the pellet, the cells were resuspended in 200µl of incomplete RPMI with heparin/0.5%BSA (heparin concentration 0.1-

1mg/ml depending on parasite line) and 0.2µl of CellTrace™ Oregon Green™ 488 (Carboxylic Acid Diacetate, Succinimidyl Ester, Invitrogen™, C34555), then incubated at room temperature on a 37cm diameter rotating wheel for 20 minutes at 30rpm. CellTrace™ Oregon Green™ 488 is cleaved by intracellular esterase and binds to amines with minimal reported cell toxicity (https://www.thermofisher.com/order/catalog/product/C34555). Pilot experiments showed that staining had no effect on rosetting ability. Once the 20-minute CellTrace™ incubation was complete, one ml of complete RPMI binding medium and 1.2µl of ethidium bromide were added and the tube incubated for a further 10 minutes at 37°C. The cells were then washed twice with incomplete RPMI to remove all traces of heparin then resuspended in 100µl of complete binding RPMI for every 10µl packed cell volume. The process for staining cRBC or EJ cells was the same as that described above except RPMI binding medium without heparin was used and no ethidium bromide was added.

In parallel to the infected erythrocyte preparation, $2.5x10^5$ cRBC or EJ cells were stained with CellTrace™ if required, then washed once in complete RPMI binding medium then resuspended in 10µl of complete binding medium. A control sample of donor erythrocytes was also prepared in the same way. Approximately 2µl of the purified infected erythrocytes (resulting in approximately 10% parasitaemia) were added to the cRBC/EJ and control tubes, which were then incubated at 37°C for one hour. Every 10-15 minutes the tubes were gently resuspended by flicking the Eppendorf tubes. Slides were prepared and rosetting counted as described above.

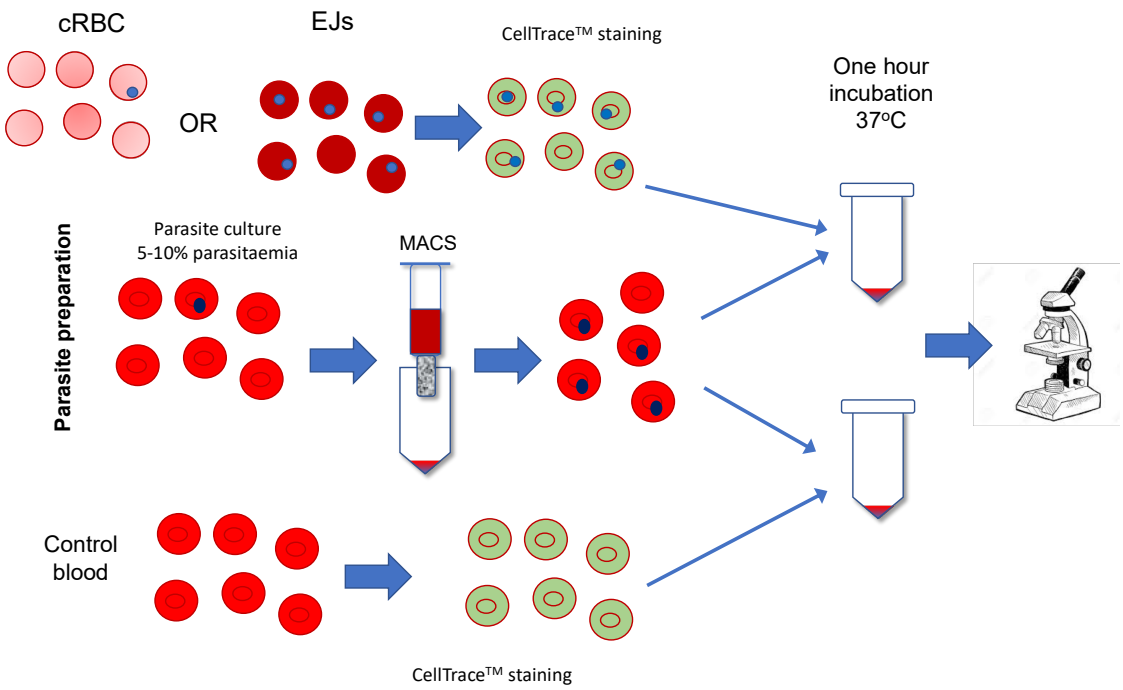


Figure 2-4 Schematic of rosetting assay with cRBC or EJ cells

2.7 Statistical analyses and production of figures

All statistical analyses were carried out using Graphpad Prism software (Version 7.0, La Jolla California, USA, www.Graphpad.com). Specific tests used are described in the methods of the relevant chapters. A p-value of <0.05 was considered to be statistically significant throughout.

Figures were created using Adobe Illustrator CC (2017.1.0 Release) and Microsoft® PowerPoint for Mac (Version 16.16, 180812). Some microscopy images were modified using ImageJ (version 2.0.0-rc-46/1.50g, http://imagej.net); details of the microscopes and cameras used along with any image manipulations have been included in the text or figure captions. The exposure times are the same for all images in a figure unless otherwise stated.

3 CHAPTER III: ARE THE GLYCOSAMINOGLYCANS HEPARAN SULFATE AND CHONDROITIN SULFATE IMPORTANT ROSETTING RECEPTORS?

3.1 Abstract

The Glycosaminoglycans are an incredibly diverse group of molecules with important roles in maintaining the extracellular matrix, cell signalling, proliferation and adhesion, both physiological and pathological. Of particular interest in the context of *P. falciparum* malaria are heparan sulfate (HS) and chondroitin sulfate (CS). In addition to their roles in hepatocyte invasion, sequestration and placental malaria, both HS and to a lesser extent CS have been proposed as rosetting receptors, though the evidence for both is somewhat limited. The results of a screening glycan microarray added weight to the hypothesis that both HS and CS might be important rosetting receptors and I set out to investigate this further using both a “block” (disrupting rosettes with heparin) and “chop” (enzymatic cleavage of HS or CS) approach with a panel of laboratory adapted parasites. While rosettes were disrupted by heparin in all parasite lines tested, I found that treatment of erythrocytes with carefully validated enzymes (Heparinase III and Chondroitinase ABC) had no effect on rosetting, suggesting that neither HS nor CS were specific host erythrocyte rosetting receptors. The binding of recombinant PfEMP1 domains from rosetting parasites was also unaffected by enzymatic treatment of uninfected erythrocytes. In addition, we found no evidence that mature erythrocytes actually express either HS or CS, though HS can be detected on early haematopoietic stem cell erythroid precursors. These data suggest that, contrary to current dogma in the rosetting field, HS is not a rosetting receptor on the uninfected erythrocyte surface and I also find no evidence to suggest that CS has a role in rosetting.

3.2 Introduction

3.2.1 Glycosaminoglycans and proteoglycans

The glycosaminoglycans (GAGs) are a diverse group of carbohydrate molecules composed of repeating disaccharide units which form large, linear, unbranched, polysaccharide chains (Gandhi and Mancera, 2008). There are four GAG subgroups (Gandhi and Mancera, 2008, Caterson, 2012, Kamhi et al., 2013, Alberts et al., 2014):

1. ***Hyaluronan (hyaluronic acid or hyaluronate, HA)-*** MW 4-8000kDa, the simplest but largest GAG with 25,000 non-sulfated disaccharide units consisting of glucuronic acid and N-acetylglucosamine. Different to other GAGs as it is not attached to protein (i.e. not a proteoglycan) and is unsulfated.
2. ***Chondroitin/dermatan sulfate ABCDE (CS)***- MW 5-50kDa, a *Galactos*aminoglycan. The disaccharide units consist of N-acetyl galactosamine and glucuronic or iduronic acid. The types, A-E, differ in their disaccharide repeats and sulfation pattern (A= GlcAβ1-3GalNAc4S, B=IdoAα1-3GalNAc4S, C= GlcAβ1-3GalNAc6S, D= Glc2Sβ1-3GalNAc6S and E= GlcAβ1-3GalNAc4S, 6diS). Dermatan, previously considered a separate subgroup, is now known as Chondroitin B.
3. ***Heparin and heparan sulfate (HS)-*** MW 10-70kDa, *Glucos*aminoglycans. The disaccharide units consist of N-acetyl-glucosamine and glucuronic or iduronic acid with variable sulfation patterns. Heparin is a distinct form of heparan sulfate which is found exclusively within mast cells and is very highly sulfated.
4. ***Keratan sulfate (KS)***- MW 4-19kDa. The disaccharide units consist of galactose and N-acetylglucosamine.

A schematic of the structure of the GAG subgroups, taken from (Caterson, 2012) is shown in
Figure 3-1.

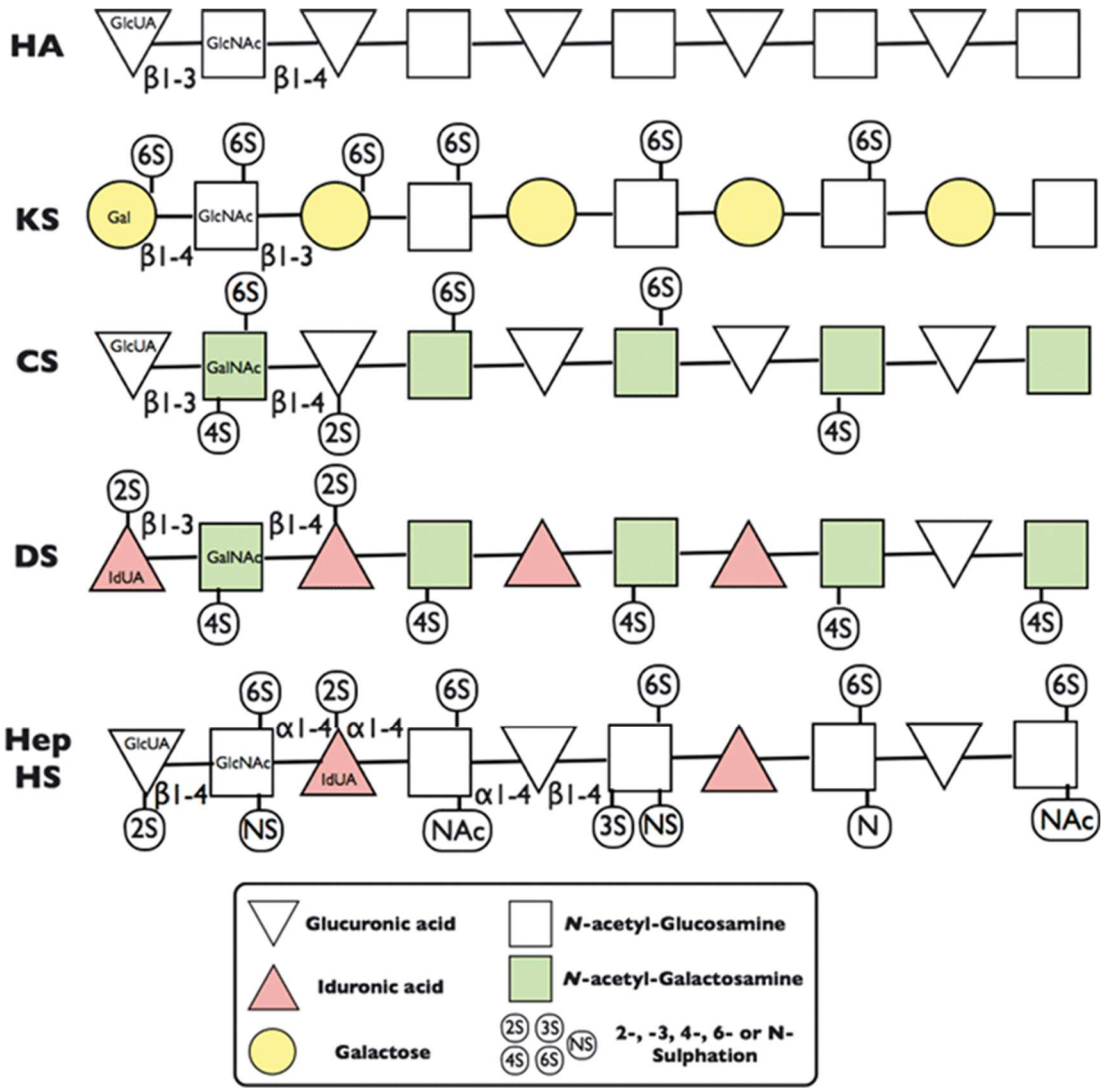


Figure 3-1 Schematic taken from (Caterson, 2012) showing the structure of the GAG subgroups. HA = Hyaluronan, KS = Keratan sulfate, CS = Chondroitin sulfate, DS = Dermatan sulfate (now considered to be a chondroitin sulfate B), Hep/HS = Heparin/heparan sulfate.

The majority of GAGs, with the exception of hyaluronan, exist as proteoglycans, consisting of a ‘core’ protein with attached GAG chains (Figure 3-2). The core protein can have a molecular weight of 10 to >600kDa (Alberts et al., 2014), and a hugely variable number of attached GAG chains

ranging from one (decorin) to over a hundred (aggrecan) (Alberts et al., 2014, Iozzo and Schaefer, 2015). The core protein can also support more than one type of GAG chain and, in the case of the Glypicans, may be linked to the cell membrane via a glycosylphosphatidylinositol anchor (Iozzo and Schaefer, 2015). For example, the syndecan family, which are amongst the best characterised proteoglycans, have a core protein of 31-45kDa and carry 1-3 GAG chains which can be both HS and CS (Varki et al., 2009). This, combined with the variation in sulphation of the GAG chains, makes for an extremely diverse range of molecules (Iozzo and Schaefer, 2015).

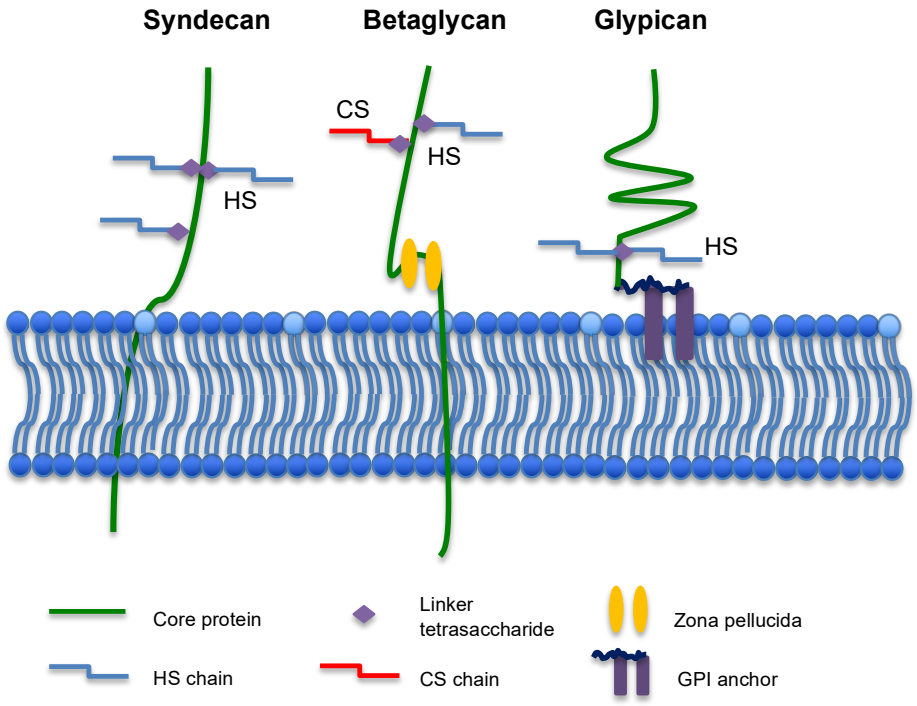


Figure 3-2 Examples of cell surface proteoglycans. Adapted from (Iozzo and Schaefer, 2015).

The GAGs are negatively charged and hydrophilic with a tendency to adopt a large volume in solution (Gandhi and Mancera, 2008). It is therefore unsurprising that they are found in the extracellular matrix of connective tissue, synovial fluid, cartilage, tendons, ligaments and the vitreous humour of the eye (Gandhi and Mancera, 2008). Chondroitin sulfate is also present in blood vessels, including the aorta, skin and the heart valves (Gandhi and

Mancera, 2008). However, the GAGs offer more than just cushioning for joints; they are also involved in cell signalling, proliferation (as co-receptors for fibroblast growth factors), inflammation and adhesion (Gandhi and Mancera, 2008, Iozzo and Schaefer, 2015). Mutations affecting GAG biosynthesis, particularly HS and CS, results in serious, multi-system pathology including Ehlers-Danlos syndrome, hereditary multiple exostoses, Simpson-Globai-Behmel syndrome and other chondrodysplasias (Mizumoto et al., 2013, Iozzo and Schaefer, 2015).

In addition to their important physiological role, a wide range of pathogens are thought to utilise GAG binding either to invade, or as a co-receptor for invasion. These range from viruses such as Cytomegalovirus, Dengue, Herpes simplex and Ebola (Chen et al., 2008, Kamhi et al., 2013, Tamhankar et al., 2018), bacteria such as *Bordetella pertussis, Chlamydia, Helicobacter pylori* and *Mycobacterium tuberculosis*, and parasites including *Toxoplasma gondii* and *P. falciparum* (for a complete list of GAG-pathogen interactions see (Chen et al., 2008, Kamhi et al., 2013). These pathological adhesion phenotypes make the GAGs particularly interesting candidates to study in the context of rosetting. For reasons discussed below, including the results of a GAG screening microarray (section 3.2.5), I chose to specifically focus on heparin/heparan sulfate and chondroitin sulfate as potential rosetting receptors and the remainder of this chapter will refer to solely to these GAGs.

3.2.2 Heparan sulfate, heparin and chondroitin sulfate: Biosynthesis and expression

The initial biosynthesis of both HS and CS is similar, taking place in the endoplasmic reticulum and Golgi apparatus (Prydz and Dalen, 2000). A four sugar ‘linker tetrasaccharide’ is formed and covalently bound to the serine residue of a core protein (Figure 3-3), after which either a glucosamine or galactosamine is added to determine whether the chain will become HS or CS respectively (Prydz and Dalen, 2000, Mikami and Kitagawa, 2013). The

chain is then extended and further modified through sulfation and/or acetylation giving rise to a multitude of different structures. Chondroitin sulphotransferases can add a sulfate group at the 4- or 6- position of the N-acetyl-galactosamine or position 2 of the glucuronic acid of CS (Caterson, 2012, Mizumoto et al., 2013). HS can be sulfated at the 2-, 3- and 6- position and the glucosamine residue can be N-acetylated or N-sulfated (Caterson, 2012). In total, 1008 possible pentasaccharide combinations have been calculated for CS and 2916 for HS (Cummings, 2009, Caterson, 2012).

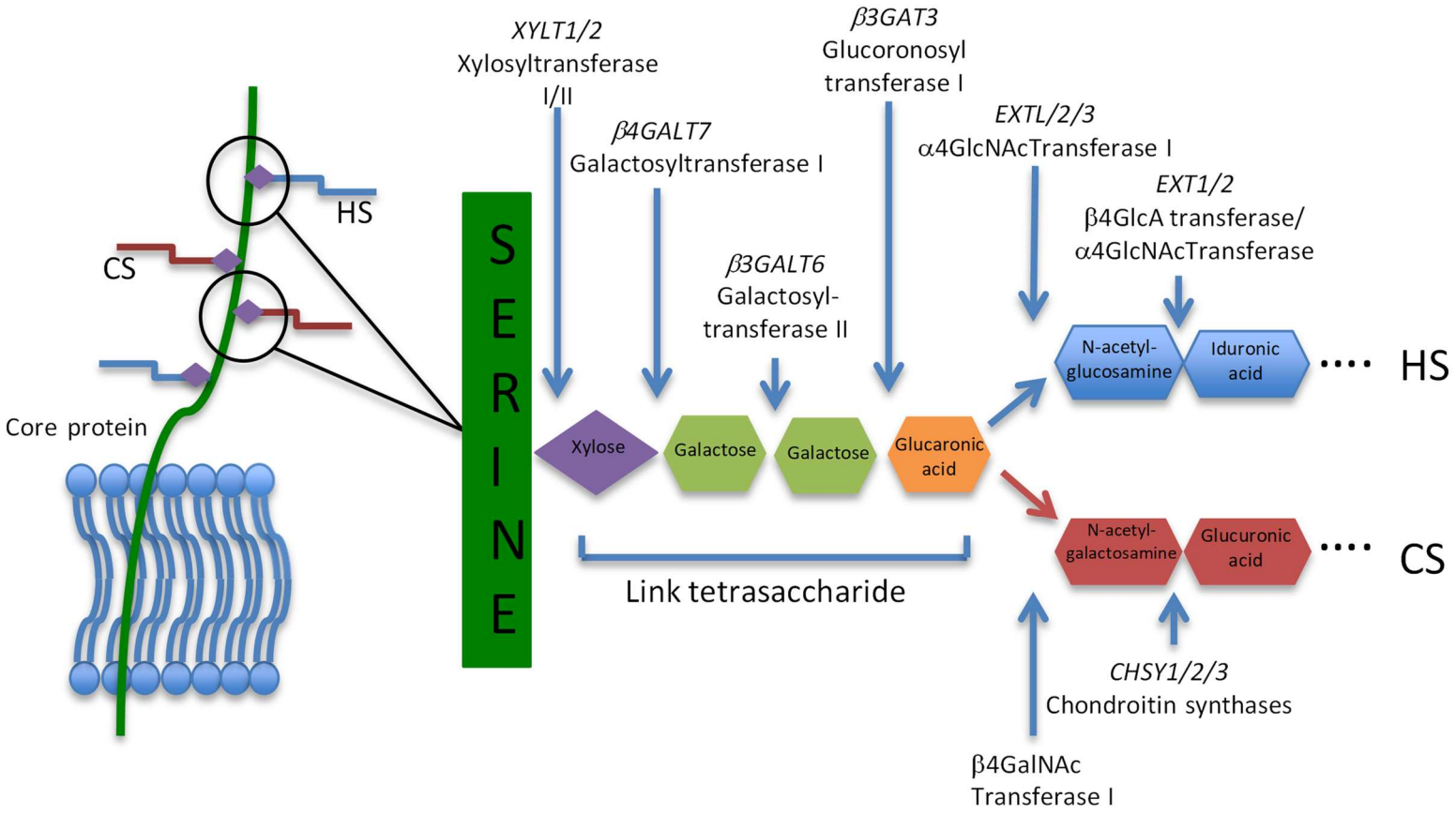


Figure 3-3 HS and CS biosynthesis. Both the coding genes and enzymes required for each link are indicated.

HS molecules can be classified according to their location as membrane-bound (syndecans, glypicans, betaglycan, neuropilin and CD44), contained within secretary vesicles (serglycin) or part of the extracellular matrix (agrin, perlecan, collagen XVIII) (Sarrazin et al., 2011). The membrane-bound forms, of particular interest here as potential rosetting receptors, are found on endothelial cells, fibroblasts, and lymphocytes (Sarrazin et al., 2011). There is a single paper which suggests that HS can also be detected on mature erythrocytes (Vogt et al., 2004), however, as will be discussed at length throughout this chapter, there are a number of issues regarding these findings.

Heparin is a unique form of HS which is of great therapeutic importance due to its anticoagulant properties (Gandhi and Mancera, 2008). Compared to HS, heparin is much more highly sulfated with 1.8-2.4 sulfate units per disaccharide compared to 0.8-1.8 for HS (Gandhi and Mancera, 2008). This gives heparin the highest negative charge of any biological molecule, and, along with other structural modifications such as a higher L-iduronic acid content, makes heparin a distinct molecule from other types of HS (Gandhi and Mancera, 2008). In addition to these biochemical differences, heparin is only found within mast cells, where it can exist as the proteoglycan serglycin (Iozzo and Schaefer, 2015). Given this very limited expression, it is unlikely that heparin itself has a role as a specific rosetting receptor, but despite this, heparin has been used as an analogue for HS in rosette disruption experiments (for example (Carlson et al., 1992)) which has led to the general assumption that HS molecules are important rosetting receptors (Mercereau-Puijalon et al., 2008, Rowe et al., 2009).

As discussed above, CS is widely expressed in various human tissues from cartilage to the aorta (Gandhi and Mancera, 2008). However, there appears to be no published evidence as to whether CS exists on mature human erythrocytes, though two papers do describe the presence of CS on rat erythrocytes (Srikanth et al., 2012, Gowd and Nandini, 2015)

3.2.3 Multiple roles for heparan sulfate and chondroitin sulfate in malaria pathogenesis

Both HS and CS have a role in multiple aspects of malaria pathogenesis. From the very beginning, sporozoites injected by the mosquito utilize HS expressed on hepatocytes to invade the liver (Frevert et al., 1993, Coppi et al., 2007, Dundas et al., 2018). Boyle *et al.* (2010) and Kobayashi *et al*. (2010) have suggested that HS-like molecules are also involved in merozoite invasion into erythrocytes. (Boyle et al., 2010, Kobayashi et al., 2010). However, both these studies rely on using heparin, rather than HS, to inhibit invasion. They demonstrate binding of heparin to their parasite ligand of interest ($MSP1_{42}$ for Boyle *et al.* and BAEBL for Kobayashi *et al.*), but do not appear to acknowledge that it could be this which is inhibiting invasion, rather than heparin blocking HS on the uninfected erythrocyte surface. There is, however, better evidence to suggest that host cell HS can act as a receptor for PfEMP1 on various endothelial cells, including human brain endothelial cells (Vogt et al., 2003, Adams et al., 2014). Large scale genetic analysis have shown a link between HS3ST3B1, a gene involved in heparan sulfate biosynthesis, and malaria resistance (Mackinnon et al., 2016), and another study has demonstrated an association between polymorphisms in the same gene and asymptomatic parasitaemia, but no data were given on any link with symptomatic or severe disease (Atkinson et al., 2012).

Chondroitin sulfate has a well-established role in the high levels of placental sequestration seen in pregnancy associated malaria (Ataíde et al., 2014) in which a specific variant of PfEMP1 known as VAR2CSA binds chondroitin sulfate A on the placenta leading to severe maternal anaemia, low birth weight babies, prematurity and significant perinatal mortality (Desai et al., 2007). Other non-VAR2CSA PfEMP1 recombinant proteins have also been showed to bind CS but it is not clear that this binding is truly representative of parasite infected erythrocyte binding *in vivo* (Resende et al., 2009).

3.2.4 Heparan sulfate and Chondroitin sulfate as potential rosetting receptors

Therefore, as the *Plasmodium falciparum* parasite has evolved to use both HS and CS at multiple points of the life cycle, it seems plausible that one or both may be involved in rosetting. A number of papers have showed that heparin and soluble HS can disrupt rosettes in about half of clinical strains *in vitro* (Udomsangpetch et al., 1989, Carlson et al., 1992, Rowe et al., 1994, Barragan et al., 1999) and inhibit binding of the rosette mediating NTS-DBL1α of Palo Alto 89F5 VarO to uninfected erythrocytes (Juillerat et al., 2011). Barragan *et al.* (2000a) showed that the size and sulfation of heparin is key in determining the rosette disruption effect. Rosettes were only disrupted by heparin fragments of 12 or more sugar units and desulfated heparin fragments had a much reduced or even no ability to disrupt rosettes (Barragan et al., 2000a). Given the level of sulfation has a significant effect on the charge of the heparin molecule, these findings perhaps suggest that rather than interacting with HS molecules on the surface on uninfected erythrocytes, heparin may exert its rosette disrupting action through electrostatic mechanisms. Alternatively, heparin may bind to PfEMP1 and block rosetting by interacting with the parasite ligands. Heparin has been shown to bind to the surface of rosetting infected erythrocytes (Barragan et al., 2000a, Heddini et al., 2001), but less so to the same non-rosetting variant (Barragan et al., 2000a) and it appears that the NTS region of the PfEMP1 is of particular importance for heparin binding (Juillerat et al., 2011).

An alternative to this relatively non-specific “block” methodology of using heparin/soluble HS to disrupt rosettes, is the use of enzymes which selectively cleave (“chop”) HS chains. Treatment of erythrocytes with Heparinase III, which selectively cleaves HS chains, offers a more specific way of ascertaining which HS on uninfected erythrocytes has a role in rosetting, assuming the enzymes have been properly validated. This sort of assay has been used in only a very few parasites strains, namely FCR3S1

(analogous to ITvar60) and TM284, which showed a reduction in rosetting after Heparinase III treated of uninfected RBCs, to approximately 20 and 30% of control respectively (Barragan et al., 1999).

The studies outlined above have led to a general acceptance that HS is a rosetting receptor, however closer scrutiny of the evidence suggests a number of potential issues. Caution must be used in interpreting data from rosette disruption assays using heparin/soluble HS as the decreased rosette frequency could be due to non-specific electrostatic effects from these highly charged molecules. The enzyme data may be more specific; however this is limited to a small number of laboratory-adapted strains, and many of the binding studies have been conducted using recombinant proteins or COS/CHO cells rather than actual parasites. Importantly, there is only one paper suggesting that HS molecules are indeed present on the surface of mature erythrocytes (Vogt et al., 2004). Heparin has previously been used as an adjunctive therapy for severe malaria but is no longer recommended due to the high incidence of bleeding complications (1986, Smitskamp and Wolthuis, 1971), though clinical trials with low- molecular weight heparin variants have taken place (Leitgeb et al., 2011, Leitgeb et al., 2017).

The evidence for CS as a rosetting receptor is much more limited. No effects were seen in studies which tested the rosette disrupting abilities of soluble CS (Rowe et al., 1994, Barragan et al., 1999), though treatment with Chondroitinase did reduce rosette frequency in one strain (TM284) (Barragan et al., 1999). Bastos *et al*. found that a fucosylated form of CS derived from sea cucumbers was able to disrupt rosettes and decrease adhesion to lung endothelial cells (Bastos et al., 2014), but the effects were lost when the highly charged, sulfated fucose branches were removed and no effect on rosetting was seen with soluble CSA. The effects of fucosylated CS on rosetting rates was similar to that of heparin, again suggesting this may be a charge related interaction, rather than specific blocking of host cell CS.

3.2.5 Oligosaccharide screening microarray results

Immediately prior to my arrival in the laboratory, Professor Rowe had collaborated with the Imperial College Glycosciences laboratory in London to investigate recombinant PfEMP1 binding to a glycan microarray in an attempt to screen for potential rosetting receptors. Recombinant PfEMP1 proteins (NTS-DBLα domains) from the parasite lines TM284 and ITvar60 were used and binding to the oligosaccharides was detected using monoclonal antibodies v2 14.1 (for ITvar60) and PH5 (for TM284). The report from Dr Yan Liu and Professor Ten Feizi (personal communication), which contained the images shown in Figure 3.4, stated that no binding was seen for the TM284 recombinant proteins. However, the ITvar60 NTS DBLα domain bound strongly to certain CS probes (14mers of CSA, CSB and CSC), and weakly to HS (Figure 3.4 and Liu and Feizi, personal communication). Binding was also seen to blood group A oligosaccharides for the ITvar60 proteins only. These findings suggested that CS could be a possible host rosetting receptor for some parasite strains and was worthy of further study.

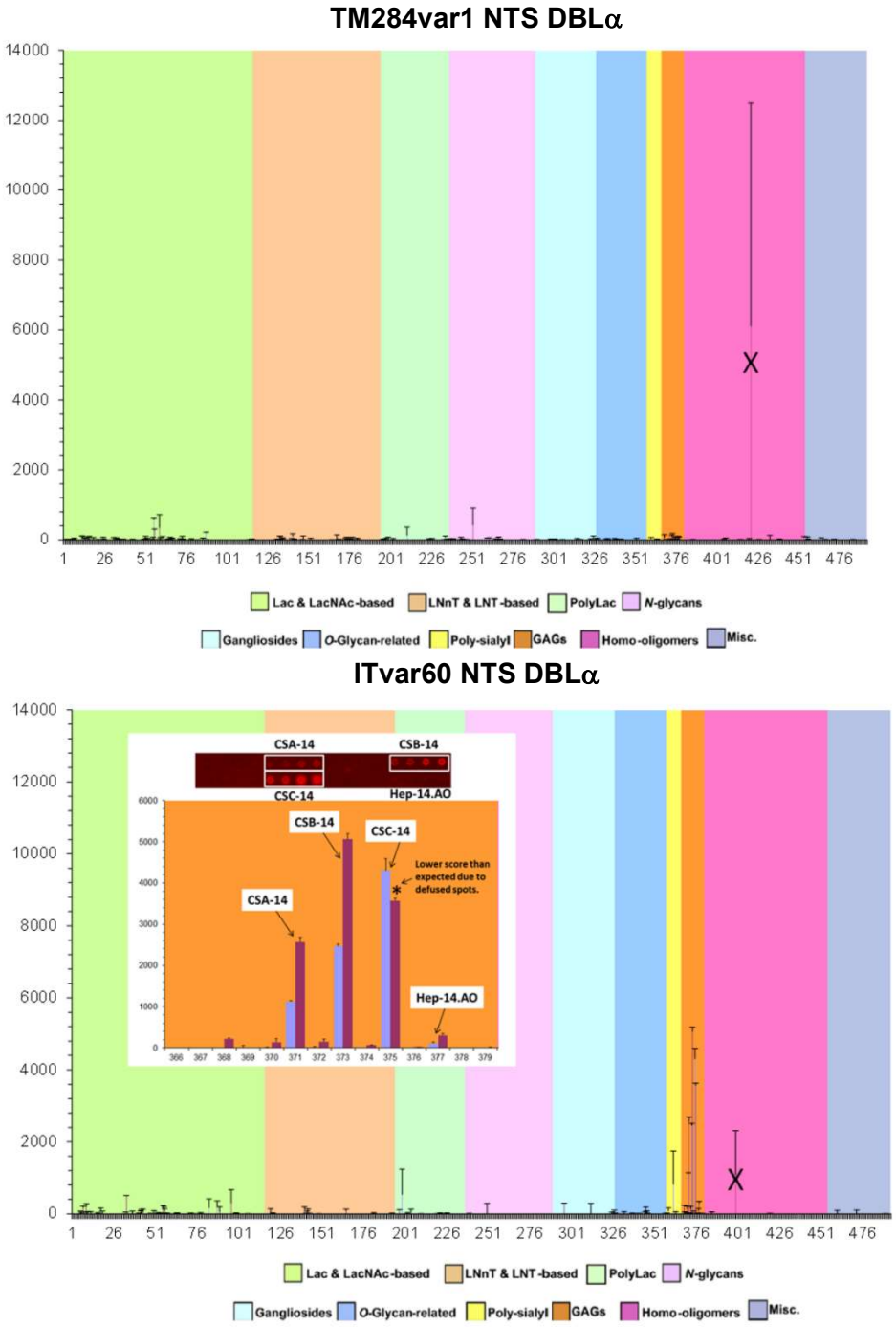


Figure 3-4 Glycan array results for TM284 NTSDBLα and ITvar60 NTS DBLα. Plots derived from Glycan array carried out by Imperial College Glycosciences laboratory showing no binding for the TM284 NTS DBLα domain (UPPER) but binding to chondroitin sulfate A, B and C, and to a lesser extent, heparan sulfate by the ITvar60 NTS DBLα domain (LOWER).

To conclude, HS and CS are attractive as potential rosetting receptors; they are known invasion receptors for multiple pathogens, *P. falciparum* itself has adapted to use both HS and CS at different points of its life cycle and there is some evidence to suggest they may be involved in rosetting. However, this evidence is problematic; it is not clear that the rosette disrupting effect of heparin or soluble HS specifically relates to blocking of the host receptors on the uninfected erythrocytes and the data using Heparinase III are limited to two parasite lines and unvalidated enzymes. The evidence that HS is present on mature erythrocytes is poor and, to my knowledge, CS on human erythrocytes has not been investigated. However, the results from the glycan microarray were sufficient to reignite our interest in both HS and CS as a potential rosetting receptor and suggested further investigation was warranted.

3.3 Hypothesis and aims

Based on the previously published evidence presented above, and the preliminary results of the screening microarray, I hypothesised that the glycosaminoglycans heparan sulfate and chondroitin sulfate are potential host rosetting receptors on human erythrocytes. The aim of this chapter was to confirm this hypothesis by providing evidence that both heparan sulfate and chondroitin sulfate are present on mature erythrocytes and that removal or blocking of these molecules has a negative effect on rosetting across different *P. falciparum* strains.

3.4 Methods

3.4.1 Rosette disruption with heparin

As described in the introduction, heparin is related, though not identical, to HS and has been used in multiple previous studies to provide evidence that HS is a rosetting receptor (Udomsangpetch et al., 1989, Carlson et al., 1992,

Rowe et al., 1994, Barragan et al., 1999). I therefore began by replicating and expanding on these experiments using both previously tested parasite lines and recently adapted *P. falciparum* strains to characterise their susceptibility, or otherwise, to rosette disruption using heparin.

P. falciparum parasites were cultured in human blood of blood group O as described in section 2.1, and two of the lines, ITvar60 and R29, were also separately cultured using erythrocytes of blood group A. High rosetting frequencies were maintained by selection as described in section 2.1.5. For each heparin disruption experiment, 2 ml of 2% haematocrit culture containing parasites at the mature trophozoite stage was stained with 2µl of 10 mg/ml ethidium bromide (final concentration 25µg/ml, Sigma46067) for 2 minutes at 37^{o}C. The culture was then centrifuged at 600g for 4 minutes and the supernatant removed. The pellet was resuspended, again at 2% haematocrit, with complete binding RPMI (culture medium with serum/Albumax but without bicarbonate) and 45µl aliquots were added to five 500µl Eppendorf tubes. Five µl of heparin sodium salt derived from porcine mucosa (Sigma, H4784, diluted with PBS as required) was added to aliquots to give a final concentration of 1mg/ml, 100µg/ml or 10µg/ml. Fucoidan (100µg/ml, Molekula, 9072-19-9) was used as a positive control and the same volume of PBS only as the negative control. Samples were blinded by a colleague then incubated for 30 minutes with gentle flicking every 10 minutes to ensure continued mixing. After this incubation, 1.8µl samples were pipetted onto a multispot slide (12 well, 6mm, Hendley-Essex, PH001) and 200 infected erythrocytes on two separate slides were counted and scored to give rosette frequency as a percentage. Three independent experiments were carried out for each parasite strain. Statistical analyses were carried out using one-way ANOVA with post-hoc Dunnet's test for multiple comparisons using GraphPad Prism software (version 7.0, La Jolla California, USA).

3.4.2 Summary of enzymatic cleavage approach

As discussed in the introduction of this chapter, the use of heparin to disrupt rosettes cannot determine whether blocking of HS on the surface of the uninfected erythrocytes is the specific mechanism of action. In order to resolve this issue, the erythrocytes in culture can be enzymatically treated to remove the GAG chains of interest (HS or CS) and the effect on rosetting observed. However, the enzymes must first be tested to ensure that they are able to remove HS or CS, particularly if no effect is seen on rosetting. The enzymes used in this study were validated using COS cells (which constitutively express both HS an CS), treated with either the enzyme or a mock treatment. The cells were then labelled with either an antibody to the whole HS/CS molecule or one directed at the stub molecule which is revealed by enzymatic cleavage of the GAG chain. In this way we were able to determine whether the enzymes were functioning as expected under the same conditions with which the parasite cultures were treated.

3.4.3 Enzymes

Heparinase III (from *Flavobacterium heparinum*, Amsbio, AMS 50-012-001) which degrades heparan sulfate (HS), and Chondroitinase ABC (from Proteus vulgaris, Amsbio, AMS.E1028-02), which cleaves hyaluronic acid, chondroitin sulfate (CS) and dermatan sulfate, were purchased from Amsbio. Heparinase III was used at a concentration of 0.04IU/ml at pH 7.4 and Chondroitinase ABC at 0.5IU/ml at pH 8.0 throughout.

Of note, Heparainase III (also known as heparin sulfate eliminase, heparan lyase III or heparintinase I) is one of a number of enzymes directed at HS-like molecules, the others being Heparinase I and Heparinase II. Heparinase I acts on the highly sulfated regions of heparin while Heparinase III is not active against heparin and instead cleaves the α(1-4) glycosidic bond between glucosamine and glucuronic acid only in unsulfated regions of HS

(Desai et al., 1993, Dong et al., 2012). Heparinase II has a broader range of action and can cleave both heparin and HS (Desai et al., 1993). Treatment of HS chains with Heparinase III (as opposed to Heparinase I or II) also reveals the desaturated glucuronate epitope for the antibody 3G10 (see below) (David et al., 1992).

3.4.4 Antibodies

Two antibody related approaches have been used throughout this work. The first is the straightforward use of primary antibodies to whole HS or CS chains, followed by an appropriate secondary antibody. The second involved prior treatment of the cell of interest with either Heparinase III or Chondroitinase ABC, followed by incubation with a primary antibody targeted to the 'stub' molecule which remains, and is revealed by, enzymatic cleavage of the whole molecule Figure 3-5. The advantage of this second approach is that it allows validation of the enzymes used and improves the specificity of the detection of the molecule of interest. Non-specific binding can be a problem, despite the use of appropriate isotype controls, however the comparison of mock and enzyme treated cells using these 'stub' antibodies reduces this issue and has allowed me to validate each part of the experimental set-up.

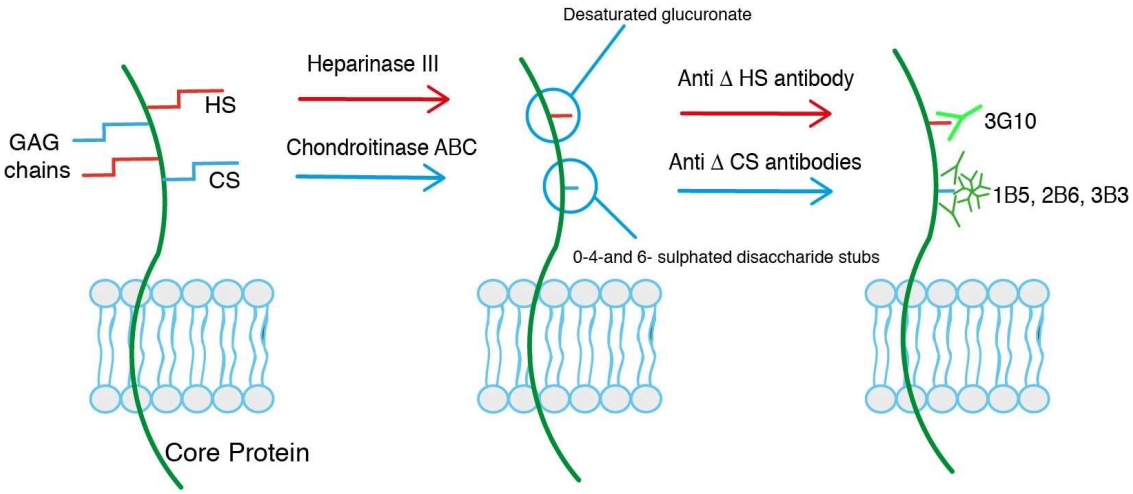


Figure 3-5 Treatment of HS and CS to reveal neoepitopes targeted by 3G10 (HS) and 1B5, 2B6 and 3B3 (CS) 'stub' antibodies

The whole molecule antibodies and their corresponding isotype controls used were; anti-heparin sulfate mouse IgM (final concentration 20µg/ml, Clone F58-10E4 Amsbio 370255-S), anti-Chondroitin sulfate mouse IgM (final concentration 16µg/ml Clone CS-56 Sigma C8035) and a mouse IgM isotype control (Thermo Scientific 02-6800). The anti- HS stub antibody, 3G10 (final concentration 40µg/ml, mouse monoclonal IgG2b,k, Clone F69-3G10, Amsbio, 370260-1), which recognises the desaturated glucuronate exposed by Heparinase III treatment was used with an IgG2b control (final concentration 40µg/ml, Abcam, ab18457). Three monoclonal antibodies to the CS stub were used; 1B5 (30µg/ml, mouse monoclonal IgG, clone 1B5, mdbioproducts, 1042014), which recognises the zero sulfated CS stub neoepitope generated by Chondroitinase digestion, 2B6 (30µg/ml, mouse monoclonal IgG, clone 2B6, mdbioproducts, 1042009), which recognises the 4-sulfated disaccharide stub and 3B3 (30µg/ml, mouse monoclonal IgM, clone 3B3, Cosmo Bio Co Ltd, PRPG-BC-M04), which recognises the 6-sulfated unsaturated disaccharide stub. For the erythrocyte experiments, an additional positive control, Glycophorin A/B (2.5µg/ml, mouse monoclonal IgG, clone E3, Sigma, G7650) was used.

Secondary antibodies used were goat anti-mouse IgG (H+L) highly cross-adsorbed secondary antibody, Alexa 488 conjugated (Life technologies, A11029), and goat anti-mouse IgM (Heavy chain) cross-adsorbed secondary antibody, Alexa 488 conjugated (Life technologies, A21042).

3.4.5 Validation of enzymes with COS cells

Due to the uncertainties regarding the presence of HS and CS on mature erythrocytes, a COS 7 cell system was used to validate both Heparinase III and Chondroitinase ABC prior to use in rosetting assays. COS 7 cells constitutively express both HS and CS. COS 7 cells were cultured as described in section 2.4 and approximately $2x10^5$ cells per well seeded onto

12mm round glass coverslips placed inside a 6 well plate the day before enzyme treatment. Once cells were 80-100% confluent, the coverslips were rinsed with incomplete RPMI for 5 minutes then removed with tweezers and placed, cell-side down, on a 50µl drop of enzyme solution (0.04IU/ml Heparinase III or 0.5IU/ml Chondroitinase ABC, diluted in incomplete RPMI at pH 7.4 or pH 8.0 respectively), or incomplete RPMI at the corresponding pH for mock treatment, on top of parafilm. The parafilm was then placed onto damp paper towels inside a petri dish to minimize evaporation during the incubation period. The COS 7 coverslips were incubated on top of the enzyme solutions for 3.5 hours at 37°C in the CO_2 incubator (Figure 3-6).

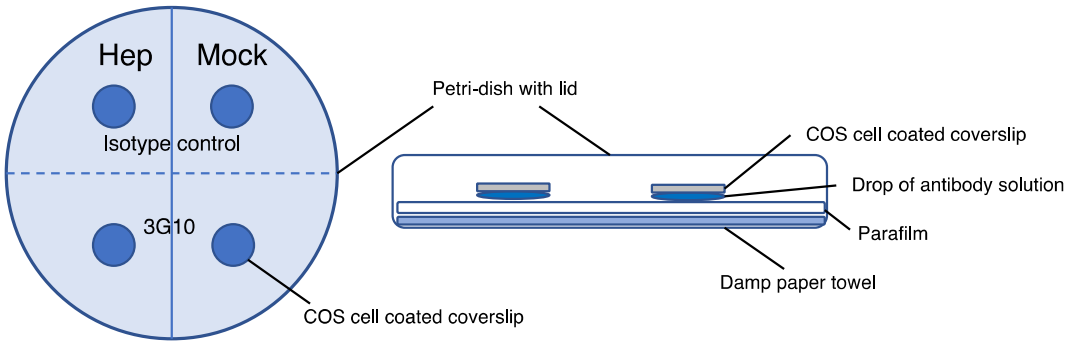


Figure 3-6 Incubation set-up for COS 7 cells on circular coverslips. The coverslips were place on top of a drop of enzyme/mock solution on parafilm on top of a damp paper towel contained within a petri dish.

The coverslips were then placed, cell side up, into a 6 well plate and washed once in complete RPMI and twice with warm PBS by gently pipetting 2ml into each well then aspirating, taking care not to disturb the coverslips. Cells were then fixed using 1ml of 2% PFA per well for 15 minutes at room temperature, followed by two further washes and blocking with 10% goat serum (Sigma, S26-M) in the same manner for 30 minutes. The primary antibodies and isotype controls detailed in section 3.4.4 were diluted in PBS/10% goat serum and 50µl drops placed on labelled parafilm as above. The COS 7 cell coverslips were then placed on top of the antibody and incubated at 4°C overnight in a humidified chamber. The following day the coverslips were carefully placed on a staining rack in a trough full of PBS to wash for 5

minutes then onto a 50µl drop of the appropriate secondary antibody, containing 1µg/ml DAPI (Sigma D9542), and incubated at room temperature and protected from light. The coverslips were then washed again in PBS and mounted onto glass slides using a small drop of Fluoromount^{TM} aqueous mounting medium (Sigma, F4680) or 5-10µl of Dabco/Glycerol (2.5mg 1, 4-Diazabicyclo[2.2.2]octane, Sigma, D27802, and 0.5ml glycerol and 0.5ml PBS) followed by sealing around the coverslip edge with nail polish.
Slides were visualized using a Leica DM LB2 fluorescent microscope or the Zeiss Imager Z1 fluorescent microscope.

3.4.6 Enzymatic treatment of parasite cultures

Two 50µl aliquots of parasite culture at 2% haematocrit was stained with ethidium bromide as detailed in section 2.6.1 then washed twice in 1ml of incomplete binding medium at either pH 7.4 (for Heparinase III/mock samples) or pH8.0 (for Chondroitinase/mock samples). After the second wash, erythrocytes were resuspended in 45µl of incomplete binding medium of the correct pH and either enzyme (to give final concentration of 0.04IU/ml of Heparinase III or 0.5IU/ml Chondroitinase ABC), or the equivalent volume of PBS (mock treatment), was added. The samples were then blinded by a colleague and incubated at 37^{o}C for 3.5 hours with regular flicking to resuspend the erythrocytes. After the incubation period, samples were washed with complete binding medium then resuspended in 50µl of complete binding medium and incubated for a further 60 minutes to allow rosettes to reform. Rosette frequency was counted using multispot slides. Three independent experiments were carried out for each parasite strain and statistical analyses were carried out using paired t-tests in GraphPad Prism software (version 7.0, La Jolla California, USA).

3.4.7 Rosette disruption after Heparinase treatment

In order to determine whether removal of HS had any impact on the ability of heparin to disrupt rosettes, 150µl aliquots of parasite culture were first treated with either Heparinase III or mock treated with PBS as detailed in section 3.4.6. The aliquots were then washed twice in complete RPMI medium and placed in a sealed incubation chamber (Modular Incubation Chamber, Billups-Rothenberg, MIC-101, www.brincubator.com). Damp paper towels were placed in the bottom of the chamber to maintain humidity and the chamber was gassed by flowing through 1%O_2/3%CO_2/Nitrogen gas mix for 2 minutes with the exit port open, then sealing the exit port of the chamber and gassing the chamber for a further 15 seconds. Samples were incubated at 37^{o}C for 1 hour to allow rosettes to reform. The treated aliquots were then stained with ethidium bromide and the heparin disruption experiment carried out as detailed in 3.4.1. These experiments were carried out using ITvar60 cultured in either blood group O or A, as these parasites had exhibited differing susceptibilities to heparin disruption depending on the blood group used to culture. Three independent experiments were carried out for each blood group.

3.4.8 Recombinant PfEMP1 domain binding to enzyme treated uninfected erythrocytes

The recombinant PfEMP1 domains and rabbit polyclonal antibodies used in these experiments had previously been generated by Dr Ashfaq Ghumra and others in the Rowe laboratory as described in (Ghumra et al., 2011, Ghumra et al., 2012).

Uninfected O positive erythrocytes from Scottish blood donors were filtered and washed as described in section 2.1.3 then resuspended at 20% haematocrit in 50µl of incomplete binding RPMI. The uninfected erythrocytes were then treated with either Heparinase III, Chondroitinase ABC or PBS

mock treatment using the same protocol as the parasite cultures in section 3.4.6. After enzyme or mock treatment, samples were washed with complete RPMI medium, then incomplete RPMI medium and resuspended with 500µl PBS to give a haematocrit of 2%. These diluted samples were washed twice in PBS and finally resuspended in PBS/0.1% IgG free BSA (Sigma, A0336) before being aliquoted into 1.5ml Eppendorf tubes, 45µl per tube. Five microlitres of protein as detailed in Table 3-1 were added to pairs of enzyme and mock treated samples giving an approximate final recombinant PfEMP1 domain concentration of 100µg/ml. Tubes were incubated on ice for one hour with regular resuspension by gently flicking the tubes. Samples were then washed twice with 750µl of PBS and resuspended in 45µl PBS/0.1% IgG free BSA before adding 5µl of rabbit polyclonal antibody giving final antibody concentration of 100µg/ml. Samples were again incubated on ice for 45 minutes, washed twice with PBS then incubated for another 45 minutes on ice with 50µl of goat anti-rabbit IgG Alexa Fluor 488 secondary antibody (1: 1000, Invitrogen, A11034). After secondary incubation, samples were washed twice with PBS then resuspended in 500µl 0.5% paraformaldehyde (PFA) diluted in PBS for 30 minutes on ice then gently centrifuged using the microcentrifuge. The PFA containing supernatant was removed and cells resuspended in 750µl of FACS buffer (PBS/0.5% BSA with 0.01% Sodium azide, Sigma, S2002) before being transferred to 5ml round-bottomed polystyrene Falcon™ tubes (Fisher Scientific, 352052). Samples were run on a Becton-Dickinson LSR II flow cytometer (BD Biosciences), counting 100,000 events per sample, and analysed using FlowJo software (version 10 FlowJo LLC, Oregon). Between three and five independent experiments were carried out for both Heparinase III and Chondroitinase ABC, each with a different blood donor.

Table 3-1 List of recombinant PfEMP1 domains and antibodies used in uninfected erythrocyte binding experiments

Recombinant protein	Primary antibody (rabbit number)	Secondary antibody
HB3var3 NTS DBL1α (negative control)	Anti-Didomain (HB3var3) (6584)	Goat anti-rabbit IgG Alexa Fluor-488 1:1000 (Invitrogen, A11034)
ITvar09 NTS DBL1α	Anti-NTS DBL1α (ITvar09) (5776)	
ITvar09 didomain (NTS DBL1α + CIDR)	Anti-NTS DBL1α (ITvar09) (5776)	
ITvar09 NTS DBL2γ	Anti- DBL2γ (ITvar09) (4772)	
ITvar09 NTS DBL4δ	Anti- DBL4δ (ITvar09) (6202)	
ITvar60 NTS DBL1α	Anti-NTS DBL1α (ITvar60) (6217)	
ITvar60 didomain (NTS DBL1α + CIDR)	Anti-NTS DBL1α (ITvar60) (6217)	
TM284 NTS DBLα	Anti-NTS DBL1α (TM284) (6219)	
None (no protein control)	Anti-NTS DBL1α (ITvar60) (6217)	

3.4.9 Immunofluorescence assay and flow cytometry for GAGS on uninfected erythrocytes and erythroid precursors

Thus far, the enzyme experiments had found no evidence to support the theory that HS or CS are important rosetting receptors. I therefore decided to challenge the basic assumption that HS and/or CS are even present on mature erythrocytes as the evidence for this is limited to a single paper for HS only (Vogt et al., 2004). Both IFA and flow cytometry were used in an attempt to detect evidence of HS and CS on mature uninfected erythrocytes. In order to try to replicate the findings of Vogt *et al.* (Vogt et al., 2004), an identical protocol to that described in their paper was used with the same

suppliers for each reagents as far as possible. This protocol was then modified to improve the specific-binding of the primary antibody and Concanavalin A staining introduced to improve visualisation of the erythrocytes. Both the Vogt *et al.* protocol and subsequent modifications are detailed below.

3.4.9.1 Immunofluorescence assay to detect HS/CS stubs on uninfected erythrocytes: Vogt et al. 2004 protocol and modifications

Blood from Scottish blood donors (Scottish National Blood Transfusion Service) was washed with incomplete RPMI as described in section 2.1.3 and diluted to a haematocrit of 2% with incomplete RPMI. Four samples of 150µl each were aliquoted into four Eppendorf tubes and washed three times with PBS. After the final wash, two of the aliquots were resuspended with 150µl of incomplete binding RPMI at pH 7.4 and the other two at pH 8.0, maintaining a haematocrit of 2%. Heparinase III was added to one of the pH 7.4 samples to give a final concentration of 0.04IU/ml and Chondroitinase ABC to one of the pH 8.0 samples (final concentration 0.5IU/ml). The remaining samples of the corresponding pH had the same volume of PBS as enzyme added as a mock treatment. The tubes were incubated at 37ºC for 3.5 hours then washed three times with PBS, separated into three further aliquots of 50µl each and incubated with primary antibody for one hour at 37ºC as follows:

Heparinase samples:

1. Mock treated Negative isotype control- Mouse IgG2b, final concentration 40µg/ml (Abcam, ab18457).
2. Heparinase treated Negative isotype control- Mouse IgG2b, final concentration 40µg/ml (Abcam, ab18457).
3. Mock treated Positive IgG control- Mouse monoclonal IgG1 Anti-Glycophorin A/B, final concentration 2.5µg/ml (Sigma, G7650).

4. Heparinase treated Positive IgG control- Mouse monoclonal IgG1 Anti-Glycophorin A/B, final concentration 2.5µg/ml (Sigma, G7650).
5. Mock treated HS stub antibody, 3G10- Mouse IgG2b, final concentration 40µg/ml (Amsbio, 370260-1).
6. Heparinase treated HS stub antibody, 3G10- Mouse IgG2b, final concentration 40µg/ml (Amsbio, 370260-1).

Chondroitinase samples:

A. Mock treated Negative isotype controls mouse IgG (Abcam, 18443) and IgM control (Serotec MCA692)- final concentration 30µg/ml.
B. Chondroitinase treated Negative isotype controls mouse IgG (Abcam, 18443) and IgM control (Serotec MCA692)- final concentration 30µg/ml.
C. Mock treated positive IgG control Gly A/B as above
D. Chondroitinase treated positive IgG control Gly A/B as above
E. Mock treated CS stub antibodies, 1B5 (Mdbioproducts, 1042014), 2B6 (Mdbioproducts, 1042009) and 3B3 (Cosmo Bio Co Ltd, PRPG-BC-M04)- each concentration 30µg/ml
F. Chondroitinase treated stub antibodies- as above.

All samples were then washed three times with PBS then resuspended with a FITC-tagged rabbit anti mouse- secondary antibody (1:25, Dako, F026102-2) for one hour at 37°C. This secondary incubation was followed by a further three PBS washes then a tertiary incubation with an Alexa 488 conjugated, rabbit anti-FITC, antibody (1:25, Molecular probes, A11090). The samples were finally washed three more times with PBS, resuspended at 20-30% haematocrit in PBS/1% BSA and smeared onto a slide. The slides were dried then a coverslip mounted on top with Fluromount™. Images were taken using a photometric evolve camera on Zeiss Imager fluorescent microscope.

Initial images showed a high level of background florescence and non-specific staining of the isotype control samples, therefore the primary

incubation period was extended to overnight (approximately 16 hours) at 4^{o}C. Concanavalin A conjugated to Alexa Fluor 594 (200µg/ml, Molecular probes, C11253) was also added to the tertiary incubation to allow for better visualisation of the red cells. Concanavalin A selectively binds to a-mannopyranosyl and a-glucopyranosyl residues on erythrocytes and was helpful in finding the correct plane for microscopic imaging.

3.4.9.2 Flow cytometry to detect HS/CS stubs on uninfected erythrocytes, cRBC and EJ cells

In addition to uninfected mature erythrocytes, two other erythroid cell types were tested for HS expression on the cell surface. These additional cell types were CD34+ haematopoietic stem cells (referred to here as cRBC), cultured as described in section 2.2 and tested at various stages of maturation, and EJ cells, an immortalised erythroid lines developed by the Duraisingh laboratory at Harvard University. Further details of both these cell types are included in Chapters 2, 4 and 5. Briefly, both cells types represent immature erythrocyte precursors, which can be matured through various bone-marrow stages of erythrocyte development, resulting in mainly early reticulocytes (cRBC), or orthochromatic erythroblasts (EJ cells).

Uninfected mature erythrocytes, cRBCs at various stages of culture, and EJ cells were Heparinase III treated as described in 3.4.6. Between 1-3x10^{5} cRBCs or EJ cells were used per antibody tested. The samples were resuspended in PBS/1% BSA and the primary antibodies added as described above followed by the appropriate secondary antibody tagged with Alexa 488 (the tertiary antibody step was not used for flow cytometry experiments). All cell types were tested for 3G10 binding after Heparinase III treatment, but the Chondroitinase ABC assay was only carried out for mature RBCs. Untreated cRBCs were assessed for binding of antibodies to the whole CS molecule, but not EJ cells.

3.4.10 Western blotting of uninfected erythrocytes/COS cells for 3G10

Having been unable to detect HS on mature erythrocytes by immunofluorescence assay or flow cytometry, additional attempts were made to identify HS using Western blotting.

3.4.10.1 Enzyme treatment and preparation of cell lysate

Uninfected erythrocytes were prepared by washing 240µl packed cell volume of mature donor erythrocytes with incomplete binding RPMI medium at pH 7.4 then resuspending in 1.2ml of incomplete binding RPMI. The cells were aliquoted into three 400µl samples. Sixteen µl of 1IU/ml Heparinase III was added to one sample (giving a final concentration of 0.04IU/ml), and 16µl of PBS added to each of the others as a mock treatment. All samples were incubated at 37^{o}C for 3.5 hours and regularly resuspended by gently flicking the tubes. Samples were then washed once with complete RPMI medium then twice with incomplete RPMI medium. The cells were thenwashed twice with PBS and lysed by resuspending in RIPA lysis buffer (Sigma, R0278, 1ml per 0.5-5x10^{7} cells), with cOmplete Mini Protease inhibitor cocktail with EDTA (Sigma, 11836153001). The cells were incubated for 20 minutes on ice then centrifuged at 8000g for 10 minutes at 4^{o}C to pellet cell debris. The supernatant was retained and frozen at -20^{o}C until use.

COS 7 cells were cultured as described in section 2.4.1 to 90-100% confluence in a T75 flask. The cells were then lifted by washing twice with PBS and adding 1ml of StemPro^{TM} Accutase cell dissociation reagent (Thermo Scientific A1110501). Accutase was used in preference to Trypsin/EDTA to allow for gentle lifting without stripping the HS proteoglycans from the cell surface. The flask containing Accutase was left at room temperature for 10 minutes until the cells began to lift then scraped with a cell scraper and washed with incomplete RPMI. The cells were resuspended in incomplete RPMI and split into two 400µl aliquots (each

containing 1.5x10^6 cells) to which was added either 16µl of Heparinase III (final concentration 0.04IU/ml) or PBS. The cells were incubated at 37ºC for 3.5 hours with constant shaking to discourage the cells from adhering. After enzyme treatment, the cells were lysed with RIPA buffer as described above, supernatant pelleted and frozen until use.

3.4.10.2 Protein gel electrophoresis

A 10% sodium dodecyl sulfate polyacrylamide gel was cast using 15mm thick plates and a 15-well comb. Solutions (sufficient to make two 15mm gels) for the lower/resolving and upper/stacking gel are listed in Table 3-2.

Table 3-2 Upper and lower gel buffers for 10% SDS PAGE gel

	Lower gel	**Upper gel**
distilled H_20	9.4ml	4.5ml
30% Bis-Acrylamide (ratio 37.5:1, Severn Biotech 20-2100-05)	7.5ml	1.9ml
10% Ammonium persulfate (Sigma, A3678)	200µl	50µl
Tetramethylethylenediamine (TEMED, Sigma T9281)	30µl	16µl
Buffer*	5.6ml	1.1ml

*Lower gel buffer consists of 1.5M TRIS base (National diagnostics, EC406) + 0.4% SDS (Sigma life science, L3771) with pH adjusted to 8.8 with concentrated hydrochloric acid. Upper gel buffer consists of 0.5M TRIS base + 0.4% SDS with pH adjusted to pH 6.8.

Glass plates were fixed into the Mini-PROTEAN® Tetra Cell casting module (Bio-Rad, 1658024) and the lower gel solution poured immediately after adding the TEMED. A small volume of 70% Ethanol was added to the top of the solution to prevent oxidation. The remaining solution in the 50ml Falcon tube was set at an angle and monitored to determine when the gel had set (approximately 15-20 minutes). Once the lower gel had set, the ethanol was poured off and TEMED added to the upper gel solution which was then immediately poured on top of the lower gel. A 15 well comb was quickly inserted and again left until the upper gel had set. The comb was then

removed and wells gently washed. If prepared ahead of time, the gels were stored flat at 4ºC for up to one week, otherwise, the gel was inserted into the electrophoresis apparatus (Mini-PROTEAN® Tetra vertical electrophoresis cell, Bio-Rad, 1658005) along with a dummy plate. The inner chamber was filled with SDS PAGE running buffer (100ml of 10x SDS buffer [0.25m TRIS-Hcl, 1.92M Glycine, 1% SDS] + 900ml dH_20) and the outer chamber half filled.

Cell lysates were defrosted and reduced by adding 3µl of 6x loading buffer (New England Biotech Gel loading dye, Purple (6X), B7024S) with 30% 2-mercaptoethanol (Sigma Aldrich, M3148) to 17µl of sample and heating at 70ºC for 10 minutes. Ten microlitres of Novex® Sharp pre-stained Protein standard (Thermo Scientific, LC5800) was added to the first well followed by 20µl of each cell lysate in the three subsequent wells. The gel was run at 125V for one hour, or until the gel front was at the bottom of the membrane.

3.4.10.3 Transfer, blocking and probing

While the gel was running, a suitably sized polyvinylidene difluoride membrane (Immobilon-P PVDF membrane, Merck Millipore, IPVH00010) was cut and activated by spraying with 70% ethanol for one minute. The membrane was then washed in transfer buffer (100ml of 10x Tris Glycine, 200ml Methanol and 700ml dH_20) along with 4 sponges and 4 similar sizes pieces of Whatman™ 3mm CHr Chromatography paper (GE Healthcare, 3030-917).

The gel was then removed from the glass plates and the transfer cassette loaded as follows: Sponge, 2x Whatman papers, gel, PVDF membrane, 2x Whatman papers and sponge. The additional sponges were placed in the dummy cassette and both cassettes were loading into the Mini Trans-Blot® Cell (Bio-Rad, 1703930), along with an ice-pack. The cell was filled with transfer buffer and run at 80V for 1 hour.

After one hour the apparatus was disassembled and the membrane stained with Ponceau S stain (Sigma life sciences P7170) for 5 minutes to confirm protein transfer. The Ponceau stain was then washed off with PBS/0.1% Tween (PBS: Oxoid, 10209252, Tween® 20, Sigma life sciences P9416) and the membrane blocked with 5% non-fat milk powder in PBS/0.1% Tween for one hour at room temperature. The membrane was then cut into strips; one mock-treated strip was incubated with anti-glycophorin A antibody as a positive control (1:1000 = 1µg/ml, anti CD236a BRIC 256, mouse IgG1, IBGRL, 9415) and the other two (mock and Heparinase III treated) incubated with 3G10 antibody directed against the HS stub (1:1000 = 1µg/ml, anti-D - heparan sulfate [3G10 epitope], purified mouse IgG2b, Amsbio, 370260-S). The antibodies were made up with 5% milk blocking solution and incubated overnight on a rocker at 4ºC. Membranes were washed three times for 5 minutes each in PBS/0.1% Tween then incubated with secondary antibody (1:8000, IRDye® 800CW Goat anti-Mouse IgG, LI-COR Biosciences, P/N 925-32210, made up in Odyssey Blocking buffer, LI-COR Biosciences, P/N 927-40000) for one hour at room temperature. After this incubation, the membranes were again washed three times in PBS/0.1% Tween then visualized on the LI-COR Odyssey scanner using the 700 and 800nm channels. Images were taken and analysed using Image Studio Lite version 5.2.5 (https://www.licor.com/bio/products/software/image_studio_lite/).

In order to confirm the presence of erythrocyte membrane proteins in lanes which were negative for 3G10, glycophorin A was used to reprobe the membranes as a loading control. The membranes were stripped using a mild stripping protocol (4g Glycine, 0.25g SDS, 2.5ml Tween in 250ml dH_20, pH adjusted to 2.2 https://www.abcam.com/ps/pdf/protocols/stripping%20for%20reprobing.pdf), washed in stripping buffer for ten minutes twice then washed with TBST twice for 5 minutes before being reblocked and incubated for 3-4 hours with

anti-glycophorin A antibody as above. Secondary antibody was then added as above and the membranes re-scanned.

3.5 Results

3.5.1 Rosetting in all parasite strains is disrupted by heparin

All the parasite lines tested were sensitive to rosette disruption by heparin to a greater or lesser extent as demonstrated in Figure 3-7, however, there were notable differences between parasite lines and, in the case of ITvar60, between cultures using different blood groups.

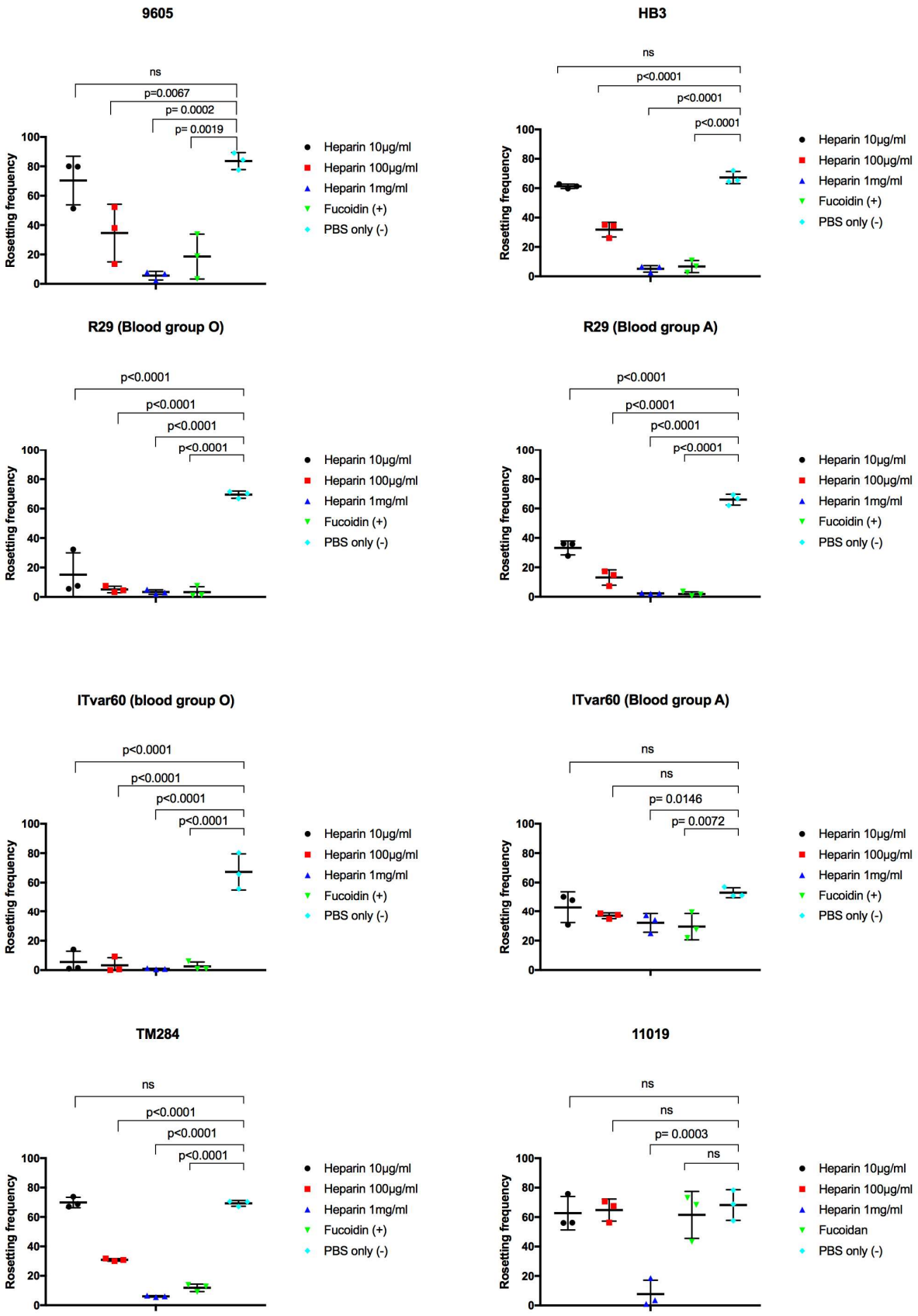

9605
ns
p=0.0067
p= 0.0002
p= 0.0019
HB3
ns
p<0.0001
p<0.0001
p<0.0001
R29 (Blood group O)
p<0.0001
p<0.0001
p<0.0001
p<0.0001
R29 (Blood group A)
p<0.0001
p<0.0001
p<0.0001
p<0.0001
ITvar60 (blood group O)
p<0.0001
p<0.0001
p<0.0001
p<0.0001
ITvar60 (Blood group A)
ns
ns
p= 0.0146
p= 0.0072
TM284
ns
p<0.0001
p<0.0001
p<0.0001
11019
ns
ns
p= 0.0003
ns
Rosetting frequency
0
20
40
60
80
100
Heparin 10µg/ml
Heparin 100µg/ml
Heparin 1mg/ml
Fucoidin (+)
Fucoidan
PBS only (-)

Figure 3-7 Rosette frequency after incubation with varying concentrations of heparin. Each graph shows the percentage rosetting frequency after treatment with different concentrations of heparin for the six parasite lines tested, with two graphs for ITvar60 and R29 showing the findings depending on the blood group of the erythrocytes used for culturing. Each individual symbol represents the results of one of three independent, blinded experiments and error bars show the mean with SD. p-values were calculated using one-way ANOVA with Dunnett's post hoc test for multiple comparisons.

Rosettes of R29, regardless of culture blood group, and ITvar60, when cultured in blood group O, were most sensitive to heparin, even at the lowest concentration of 10μg/ml. The majority of the other lines showed a statistically significant reduction in rosette frequency at 100μg/ml, and rosetting was virtually abolished by 1mg/ml of heparin in all strains. Notable exceptions include ITvar60 when cultured in blood group A, which showed a less dramatic reduction in rosette frequency even at high heparin concentrations, and 11019 which also stood out as the only line which was resistant to Fucoidan. Of note, ITvar60 has been described as having a 'preference' for blood group A, forming bigger, stronger rosettes which are more resistant to disruption, (Barragan et al., 2000b, Ch'ng et al., 2016) possibly due to the binding of the blood group A antigen to parasite-derived RIFINS (Goel et al., 2015). Such a preference is not seen for R29 in the published literature (Carlson and Wahlgren, 1992) (though some preference for Group A has been seen in our laboratory, Fennell and Rowe, personal communication), which may explain the variation in sensitivity for heparin seen for ITvar60 and not R29.

3.5.2 Validation of enzymes and antibodies with COS 7 cells

As discussed in the introduction to this chapter, the use of heparin, or indeed soluble HS or CS, to disrupt rosettes does not necessarily provide evidence that HS or CS are specific rosetting receptors. For this reason, the alternative approach of enzymatically removing HS or CS from the erythrocyte surface was used. Pilot experiments showed no obvious effect of enzymatic treatment on rosetting frequency, in contrast to the findings of Barragan *et al*. (Barragan et al., 1999), therefore before going any further, the decision was made to validate the enzymes using COS cells which constitutively express both HS and CS. Initially, the labelling of the whole HS or CS molecule was relatively poor on IFA, though a decrease in HS and CS expression could be seen after enzyme treatment (Figure 3-8 A). The effect was much more obvious when using the antibodies directed against the HS and CS 'stubs' (Figure 3-8 B). We therefore felt reassured that the enzymes were capable of

cleaving HS/CS under the same conditions (pH, temperature, duration of treatment, media) that were used in the parasite assays.

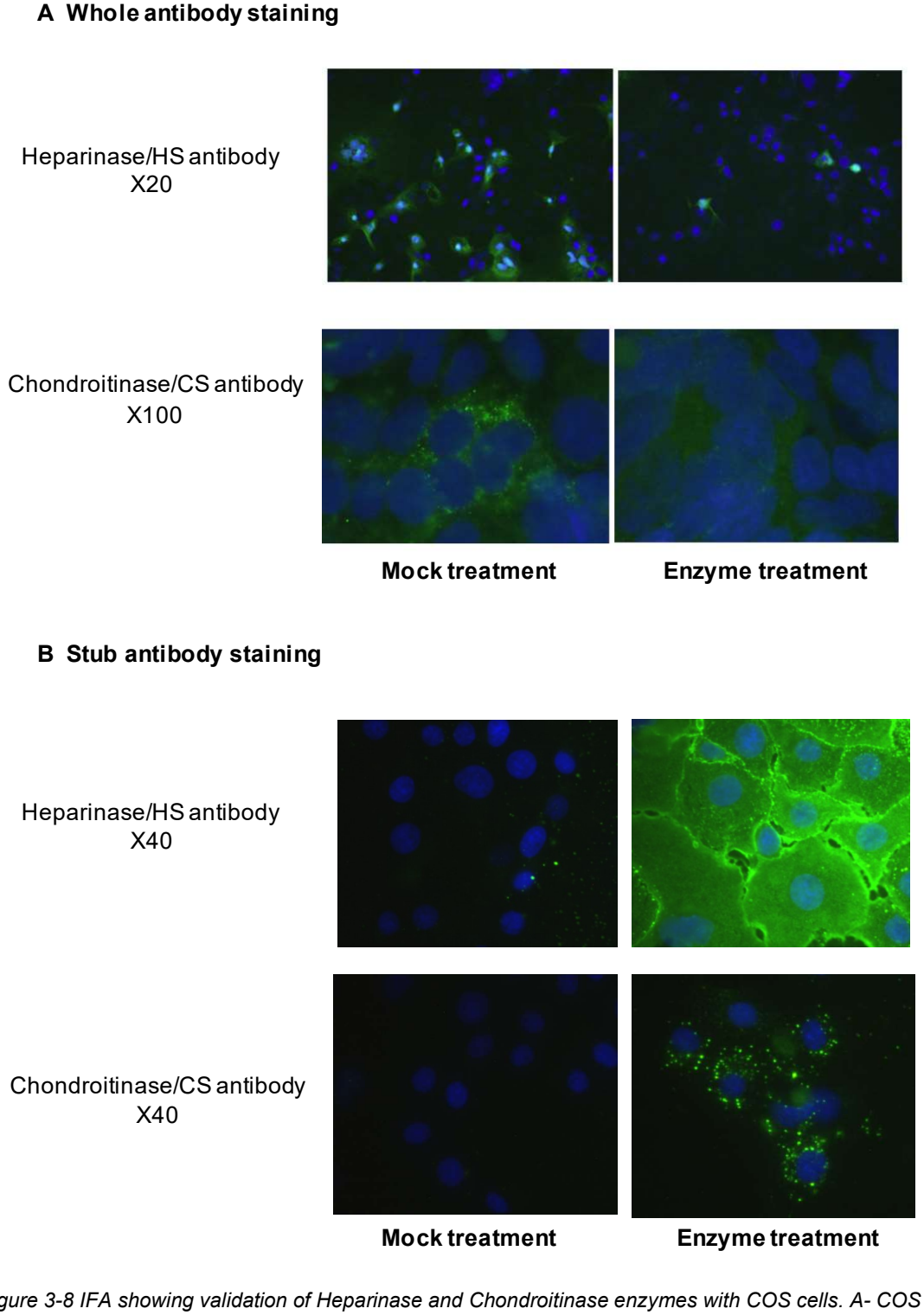


Figure 3-8 IFA showing validation of Heparinase and Chondroitinase enzymes with COS cells. A- COS cells treated with Heparinase (upper) or Chondroitinase (lower) were labelled with an antibody directed against the whole HS or CS molecule respectively. B- COS cells were again treated but labelled with antibodies directed against the stub molecules remaining after HS or CS cleavage.

3.5.3 Heparinase or Chondroitinase treatment does not reduce rosetting

Having validated the enzymes as described above, cultures of rosetting parasites containing both infected and uninfected erythrocytes were treated with either Heparinase III or Chondroitinase ABC and the rosette frequency counted as detailed in section 3.4.6. Prior to treatment, the rosette frequency of the parasites ranged from 65-89%. After treatment there were no statistically significant differences (two-tailed, paired t-test, $p>0.05$ for all strains) between mock and enzyme treated cultures (Figure 3-9). Two strains, R29 and ITvar60, were also cultured in blood group A, but again, no significant difference in rosetting was seen between treated and untreated cultures (Figure 3-9).

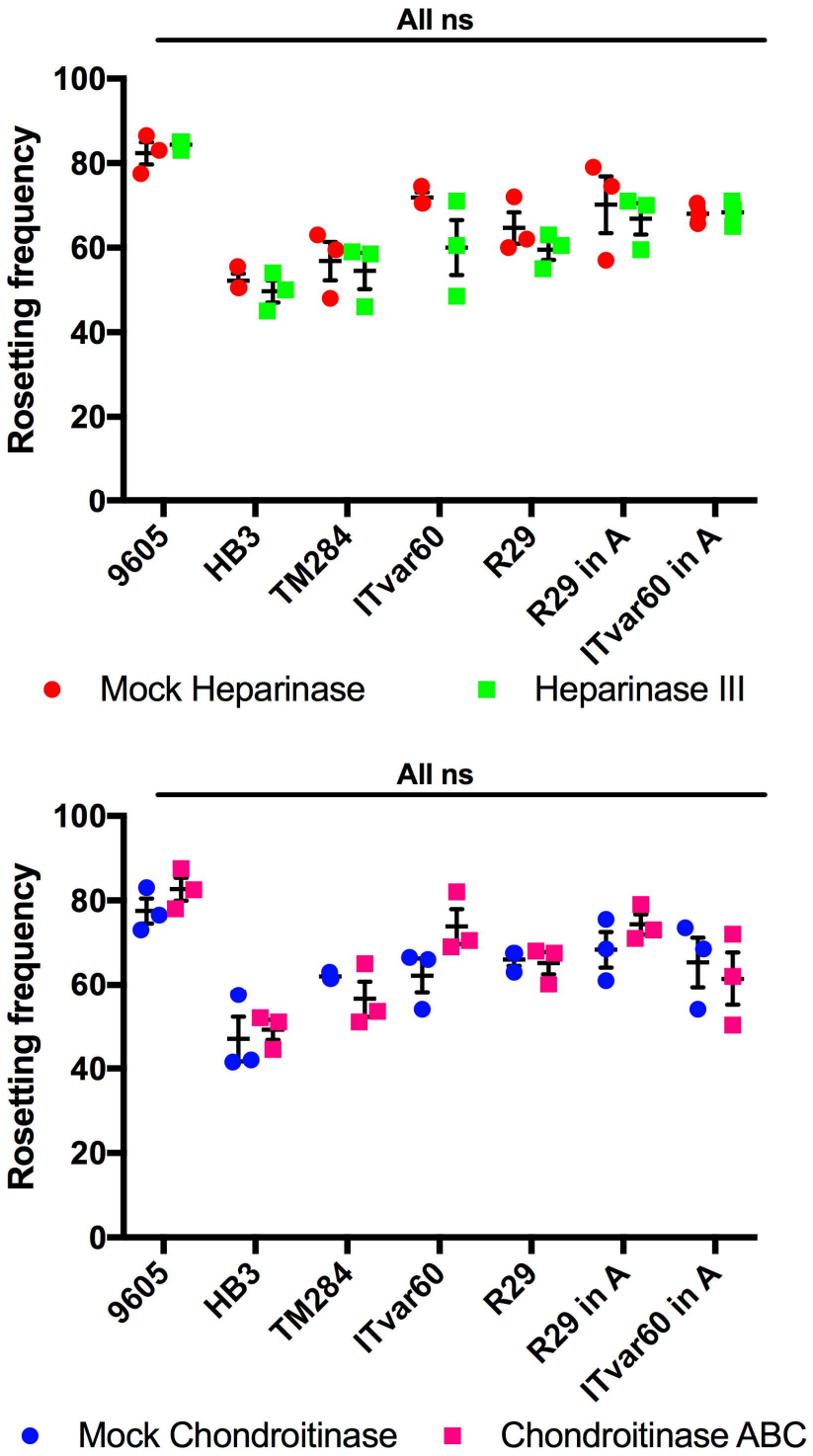


Figure 3-9 Rosetting frequency after treatment with Heparinase III (UPPER) or Chondroitinase ABC (LOWER). Each data point indicates the rosetting frequency for a single, independent experiment with circular symbols representing mock treated cultures and squares, enzyme treated. The corresponding parasite lines are shown on the x-axis. Three independent experiments were carried out for each parasite strain and error bars show the mean and SD. Two-tailed paired t-test showed no significant differences between mock and enzyme treatment for any strain ($p>0.05$ for all comparisons).

3.5.4 Ability of heparin to disrupt rosettes is not affected by Heparinase III treatment

Having found no difference in the rosetting abilities of Heparinase III or mock treated erythrocytes, the effect of pre-treatment with Heparinase III on rosette disruption by heparin was investigated. The parasite ITvar60 was chosen for these experiments and was cultured separately in blood group A and O. ITvar60 was chosen as it is demonstrated marked sensitivity to heparin when cultured in blood group O, but less in its preferred blood group, A. If the rosette disruption by heparin was due to a specific interaction with HS on the surface of the uninfected erythrocyte, it might be expected that removal of HS would decrease the effectiveness of heparin, whereas if the mechanism was non-specific e.g. related to the negative charge of heparin, no difference would be seen.

In fact, heparin was equally effective at disrupting rosettes in both the Heparinase III and mock treated erythrocytes, with rosetting significantly reduced at all concentrations of heparin in parasite cultured in both blood groups (Figure 3-10). Of note, in this experiment, the lower concentrations of heparin had more of an effect on rosetting on the parasites cultured in blood group A, than seen in the previous experiments shown in Figure 3-7.

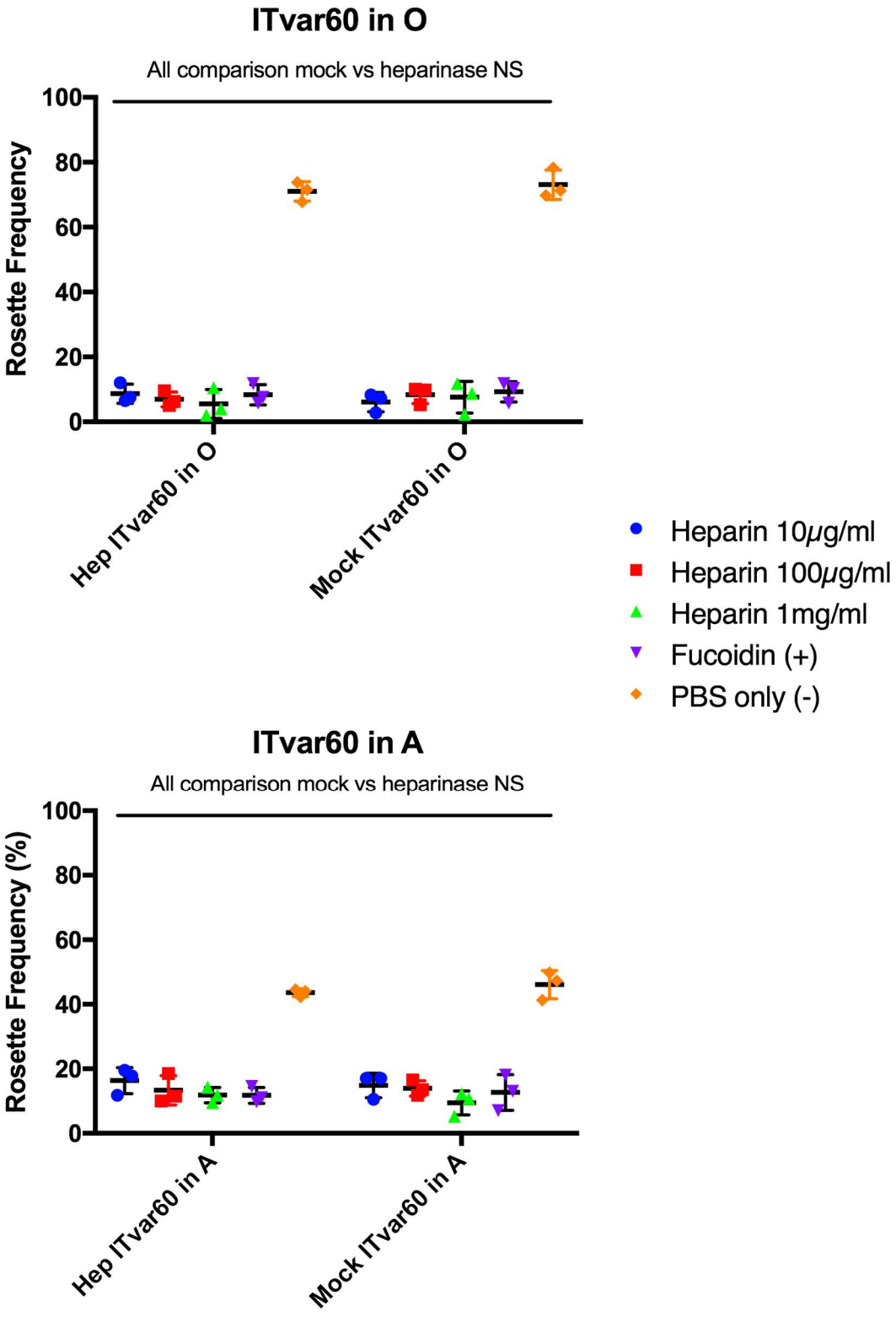


Figure 3-10 The ability of heparin to disrupt rosettes is not affected by prior treatment of the culture with Heparinase III. ITvar60 parasite cultures grown in both blood group O (upper) and A (lower) were pre-treated with Heparinase III or mock treatment, followed by the same heparin disruption experiment described previously. Each individual symbol represents the result of one of three independent, blinded experiments and error bars show the mean with SD. p-values were calculated using one-way ANOVA with Dunnett's post hoc test for multiple comparisons

3.5.5 PfEMP1 binding to uninfected erythrocytes is not reduced by Heparinase or Chondroitinase treatment

While the removal of HS and CS did not appear to have any effect on the rosetting ability of infected and uninfected erythrocytes in culture, I was interested to discover whether the binding of recombinant PfEMP1 domains was changed by Heparinase/Chondroitinase treatment. As discussed in Chapter 1, the main parasite adhesion molecule involved in rosetting in *P. falciparum* is PfEMP1, which consists of multiple Duffy binding-like (DBL) domains and cysteine-rich interdomain regions (CIDR) with the end terminal DBLα domain thought to be the most important for rosetting (Vigan-Womas et al., 2012). In order to explore which, if any, PfEMP1 domains might have a role in HS/CS binding, immunofluorescence and flow cytometry experiments were carried out assessing the binding of a panel of PfEMP1 recombinant proteins to Heparinase or Chondroitinase treated, uninfected erythrocytes. Recombinant proteins from variants ITvar09, derived from parasite strain R29, and ITvar60, were investigated. The NTS-DBLα from TM284 acted as the positive control and the non-rosetting variant HB3var3 was included as a negative control in addition to no protein controls.

IFA demonstrated binding to uninfected erythrocytes of the recombinant domains ITvar09 NTS-DBLα, ITvar09 didomain, ITvar09 DBL2γ, ITvar60 NTS-DBLα, ITvar60 didomain and subtle binding for ITvar09 DBL4δ (Figure 3-11), suggesting that multiple PfEMP1 domains may contribute to rosetting. The remaining domains did not show any binding.

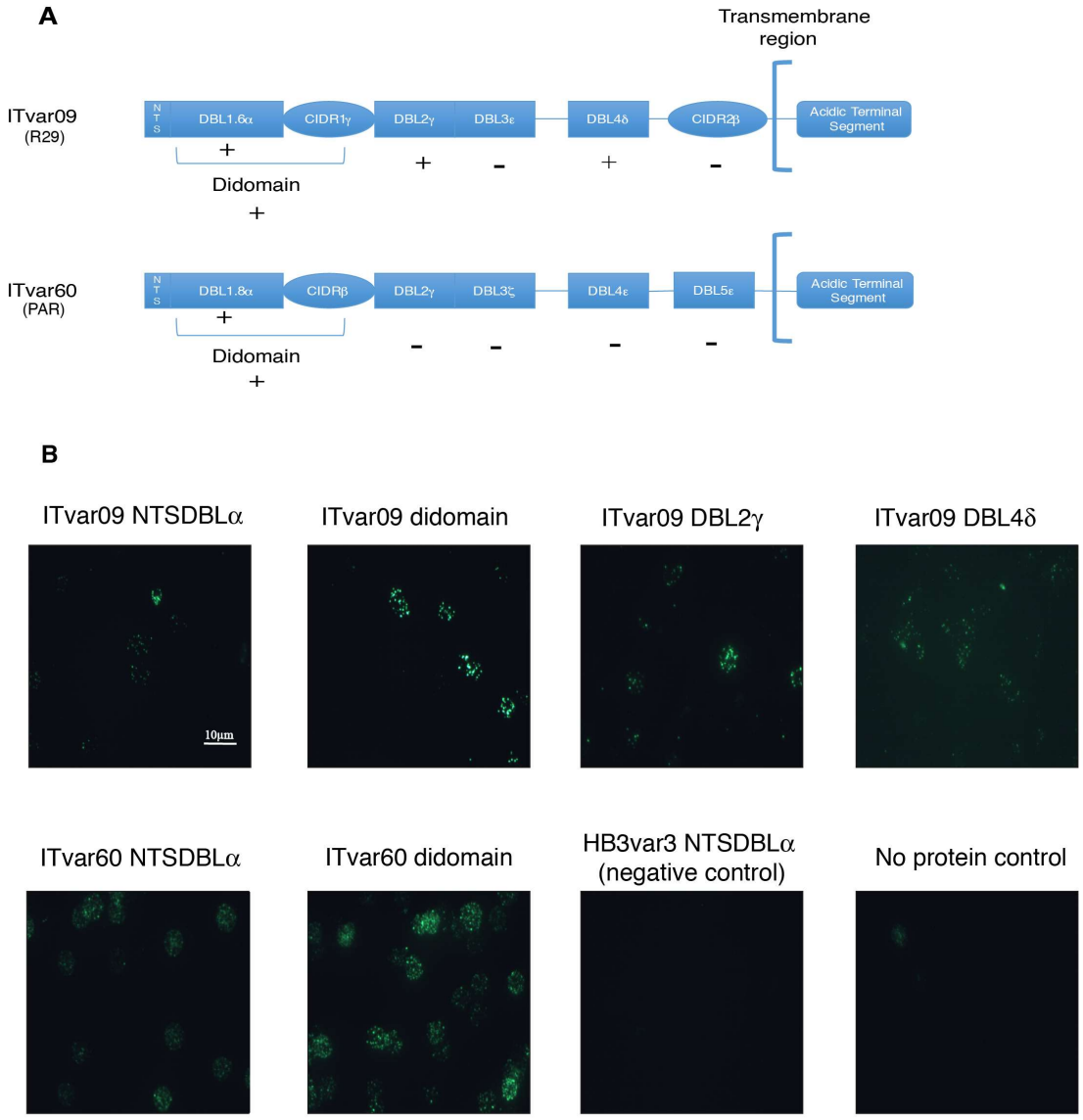


Figure 3-11 Multiple rPfEMP1 domains can bind uninfected erythrocytes. A- Schematic of PfEMP1 domains from parasites R29 and ITvar60, adapted from (Rowe et al., 1997). (+) indicates that binding to uninfected erythrocytes was observed for the individual rPfEMP1 domain and (–) that no binding was seen. B- IFA of uninfected erythrocytes with green Alexa488 fluorescence indicating binding of the rPfEMP1 domain.

Binding was not affected by either Heparinase III or Chondroitinase ABC treatment of the erythrocytes on flow cytometry (Figure 3-12) or IFA, with the exception of ITvar09 DBL2γ, which showed slightly decreased binding after Chondroitinase treatment in one donor, though this was not consistently seen with other donors or on repeat experiments.

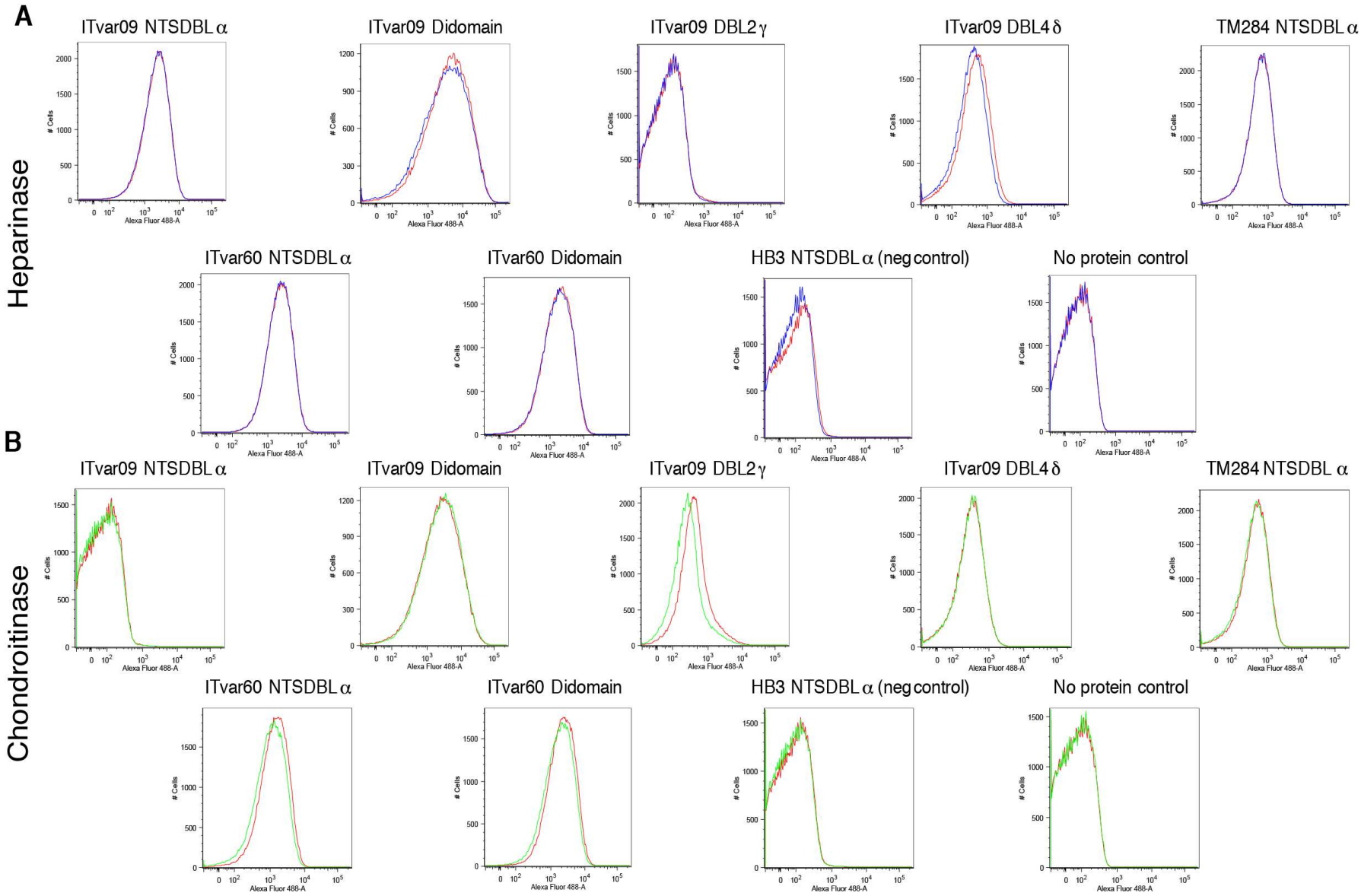

A
Heparinase
ITvar09 NTSDBLα
ITvar09 Didomain
ITvar09 DBL2γ
ITvar09 DBL4δ
TM284 NTSDBLα
ITvar60 NTSDBLα
ITvar60 Didomain
HB3 NTSDBLα (neg control)
No protein control
B
Chondroitinase
ITvar09 NTSDBLα
ITvar09 Didomain
ITvar09 DBL2γ
ITvar09 DBL4δ
TM284 NTSDBLα
ITvar60 NTSDBLα
ITvar60 Didomain
HB3 NTSDBLα (neg control)
No protein control
Cells
Alexa Fluor 488-A

Figure 3-12 Flow cytometry plots showing recombinant PfEMP1 domain binding to uninfected erythrocytes after mock or enzyme treatment. Three to five independent experiments were carried out with different donors (depending on availability of the recombinant protein domain) and a representative plot from these experiments for each domain is showed. A- Blue indicates Heparinase treated cells and red mock treated cells. B- Green indicated Chondroitinase treated cells and red mock treated.

3.5.6 Heparan sulfate or chondroitin sulfate not detected on mature uninfected erythrocytes

Thus far, these results did not support the hypothesis that HS or CS are important, specific rosetting receptors. Therefore, it was decided to reinvestigate whether these molecules are even present on mature erythrocytes. Initial experiments using antibodies to whole HS or CS were unable to detect either GAG, therefore the alternative method using enzymatic pre-treatment and antibodies to the remaining stub molecule developed by Vogt *et al.* (2004) for detecting HS was used. No definitive staining for HS or CS stubs was demonstrated when compared to the isotype control, though the background staining was similar to that shown in Vogt *et al.* (Figure 3-13).

To decrease the excessive background noise, the protocol was modified to include an overnight incubation and additional staining of erythrocytes with Concanavalin A. Cleaner images were obtained and again, there was no convincing evidence of HS or CS staining (Figure 3-14).

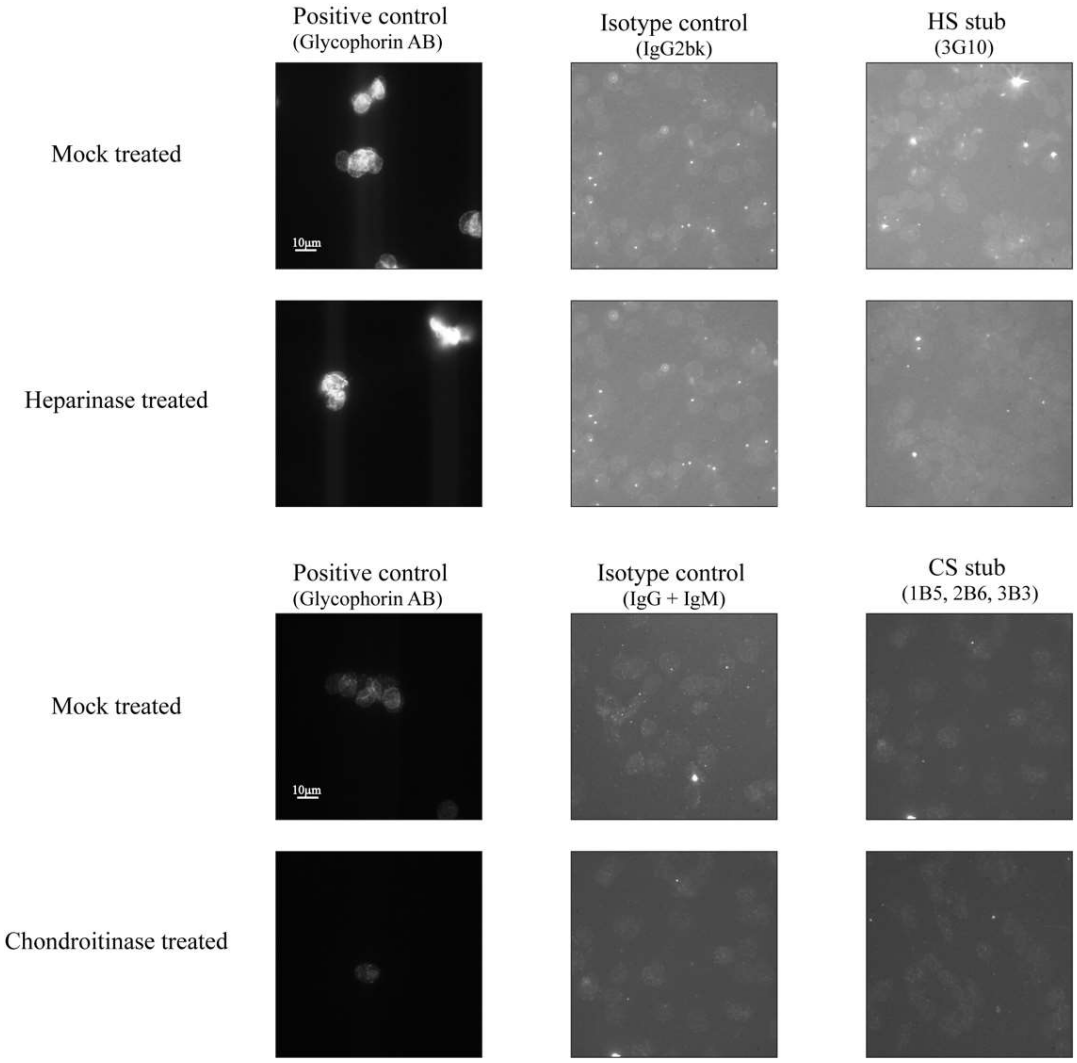


Figure 3-13 Fluorescent microscopy images showing Heparinase/mock treated cells (UPPER) and Chondroitinase/mock treated cells (LOWER) incubated with primary (3G10 for Heparinise and 1B5, 2B6 and 3B3 for Chondroitinase), secondary (Rabbit anti-mouse FITC) and tertiary (Anti-FITC Alexa 488) antibodies. Exposure time was 55msec with the exception of Anti-glycophorin AB positive control which was 1ms due to extremely bright staining and clumping of cells (hence darker background). Results are representative of three independent experiments. Images taken using photometric Evolve camera on Zeiss Imager Z1 florescent microscope.

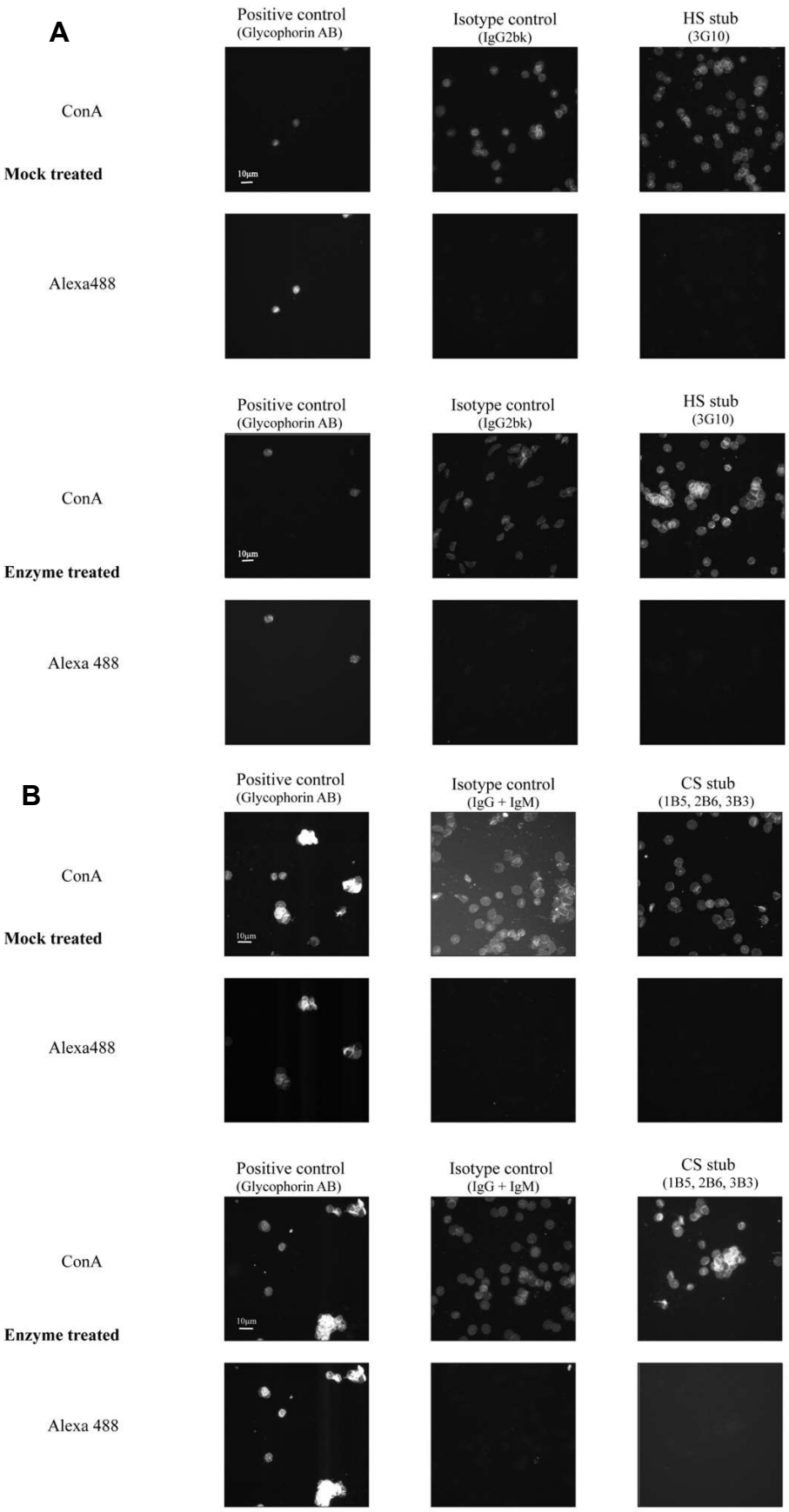

A
Positive control
(Glycophorin AB)
Isotype control
(IgG2bk)
HS stub
(3G10)
ConA
Mock treated
Alexa488
10μm
Positive control
(Glycophorin AB)
Isotype control
(IgG2bk)
HS stub
(3G10)
ConA
Enzyme treated
Alexa 488
10μm
B
Positive control
(Glycophorin AB)
Isotype control
(IgG + IgM)
CS stub
(1B5, 2B6, 3B3)
ConA
Mock treated
Alexa488
10μm
Positive control
(Glycophorin AB)
Isotype control
(IgG + IgM)
CS stub
(1B5, 2B6, 3B3)
ConA
Enzyme treated
Alexa 488
10μm

Figure 3-14 Representative images from revised protocol with overnight primary incubation (antibodies as above A- Heparinase treated cells, B- Chondroitinase treated) and additional staining of erythrocytes with Concanavalin A tagged with Alexa 594 during tertiary incubation. Identification of cells with ConA allowed for shorter exposure times (10ms for ConA images and 5ms for Alexa488) thereby reducing background noise. Again, no evidence of either HS or CS was observed.

Uninfected erythrocytes enzyme-treated and incubated as above also failed to show any evidence of HS or CS when analysed by flow cytometry (Figure 3-15).

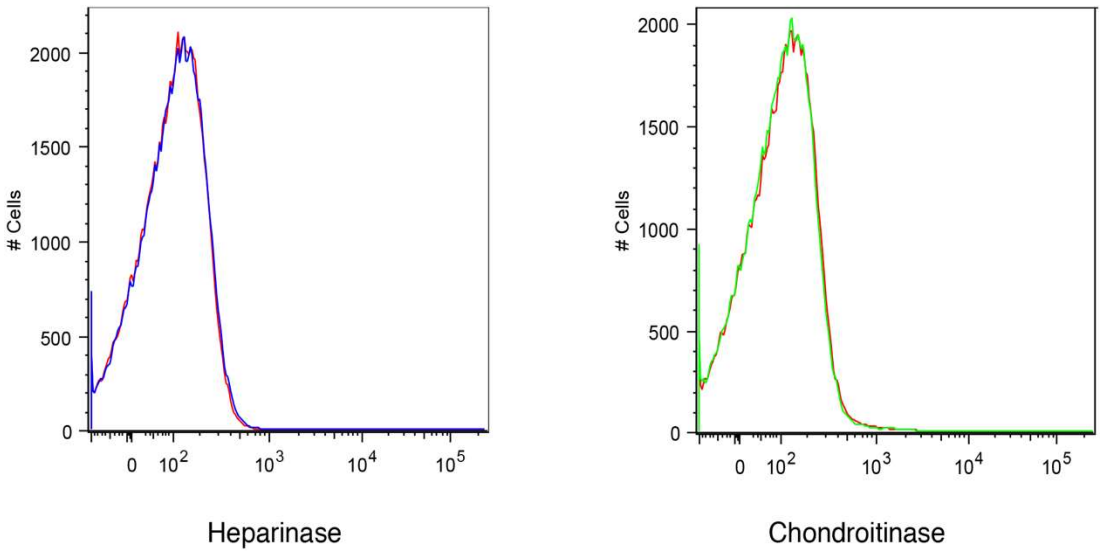


Figure 3-15 Flow cytometry of enzyme treated cells. Erythrocytes were prepared as described and analysed on a Fortessa flow cytometer, 100,000 events were counted. Blue indicates Heparinase treated cells, green Chondroitinase treated cells and red represents mock treatment.

3.5.6.1 Heparan sulfate is present on early erythroid precursors

In contrast to the above finding on mature erythrocytes, HS could be detected using a similar protocol in the early stages of culture (Day 8 and 11) of both cRBCs derived from adult bone marrow (Figure 3-16) and undifferentiated EJ cells (Figure 3-17). Of note, though the concentration of Heparinase III was the same for mature erythrocytes and the precursors cells, the number of precursor cells was much lower (as detailed in section 3.4.9.2).

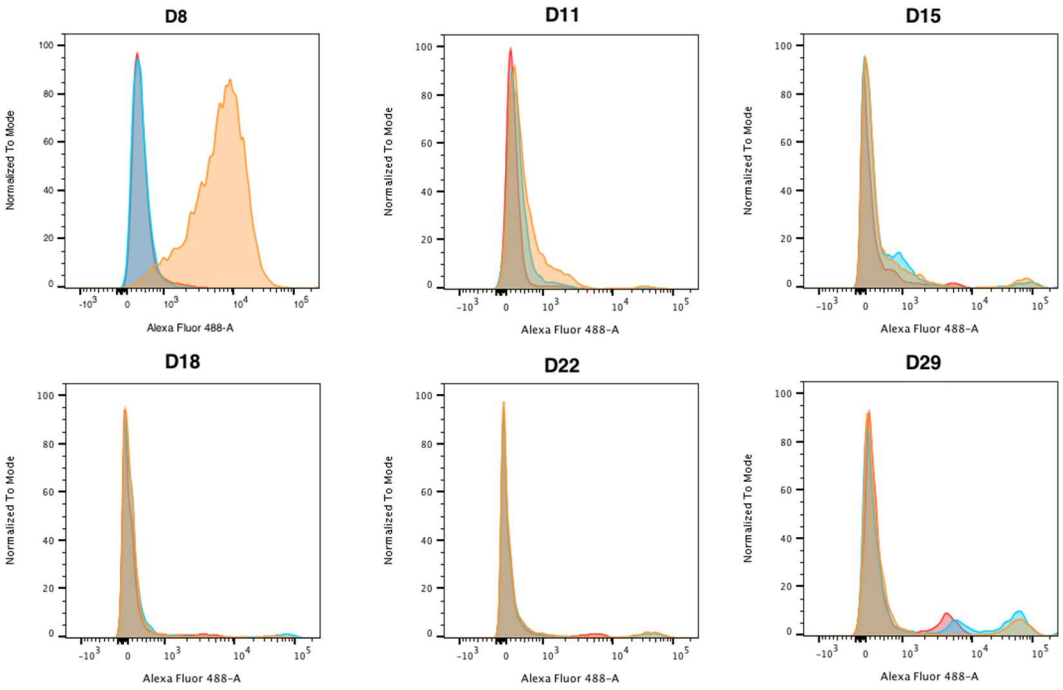


Figure 3-16 Progressive loss of HS expression with maturity of cRBC. Representative flow cytometry plots demonstrating the loss of HS expression from culture day 8 to maturity on day 18-29. Red- IgG2bk isotype control, Blue- mock treated cRBC incubated with the 3G10 antibody to the HS stub followed by Alexa488 tagged secondary antibody, Orange- Heparinase III treated cRBC labelled as per mock cells. Gating strategies are shown in Appendix 1. Of note, the apparently 'positive' signal in a minority of cells at D29 relates to disintegrating and dying cells, See figure Figure 5-10 for further details).

HS was progressively lost as the cells matured and was undetectable in either cRBC or EJs cells in their matured stage (after approximately 18 or 8 days in culture respectively).

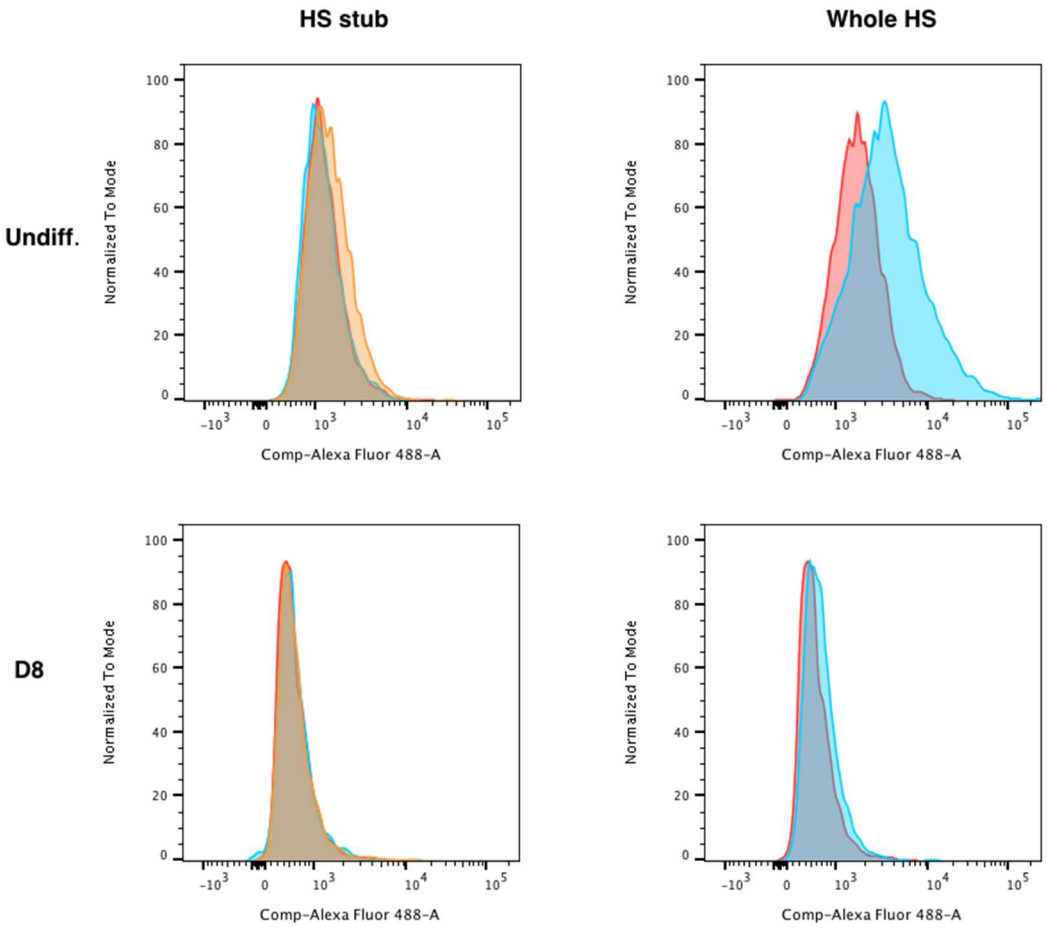


Figure 3-17 HS expression is lost as EJ cells differentiate. HS can be detected on undifferentiated EJ cells (approximately equivalent to D10 of cRBC culture) using both the 3G10 HS stub antibody on Heparinase III treated cells, and an antibody directed against the whole HS molecule (UPPER panel), however this is lost once the cells mature (LOWER panel). Red- isotype control, Blue- mock treated cells with 3G10 stub antibody or whole HS antibody, Orange- Heparinase treated cells with 3G10 stub antibody.

Further details of the cRBC and EJ cells are discussed in depth in later chapters. Of note, even in their most mature state, cRBC and EJ are probably equivalent to erythrocytes which are still contained within the bone marrow and therefore these data support my previous findings that HS is not detectable in peripheral circulating erythrocytes though it is present on very early bone marrow erythroid precursors and is likely lost during maturation.

Similar experiments were carried out with cRBC tested using the whole CS antibody and in keeping with the above data, no evidence of CS was seen (Figure 3-18).

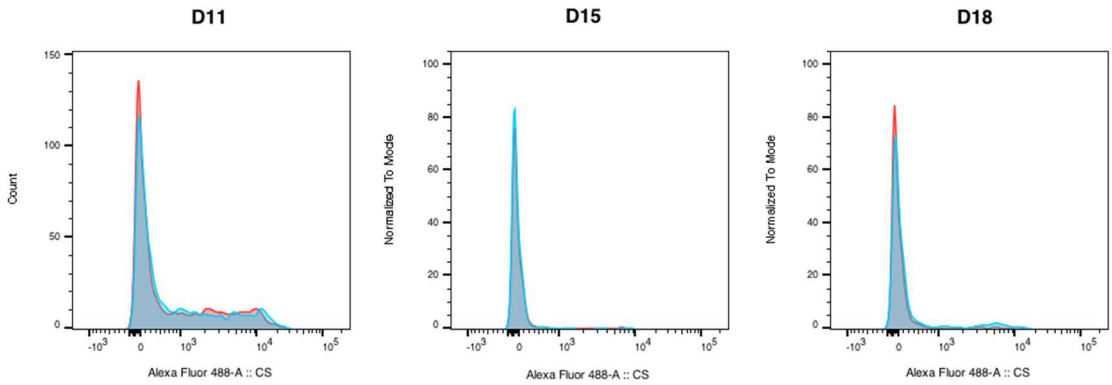


Figure 3-18 Flow cytometry plots showing no evidence of CS on cRBC. Red- IgM isotype control, Blue- antibody to whole CS.

3.5.6.2 Western blotting detects heparan sulfate on COS 7 cells but not mature erythrocytes

As no HS could be detected on the mature erythrocyte surface using the methods detailed above, it seemed prudent to use an alternative detection method, such as Western blotting. While any HS detected could not be localized to the cell surface, a negative result would further validate the findings of the IFA and flow cytometry experiments carried out thus far.
Gel electrophoresis, protein transfer and probing for glycophorin A on mature erythrocytes was successful as demonstrated in Figure 3-19 by the Ponceau staining and positive glycophorin A bands. However, no evidence of the HS stub was seen when comparing the mock and Heparinase III treated mature erythrocytes (Figure 3-19).

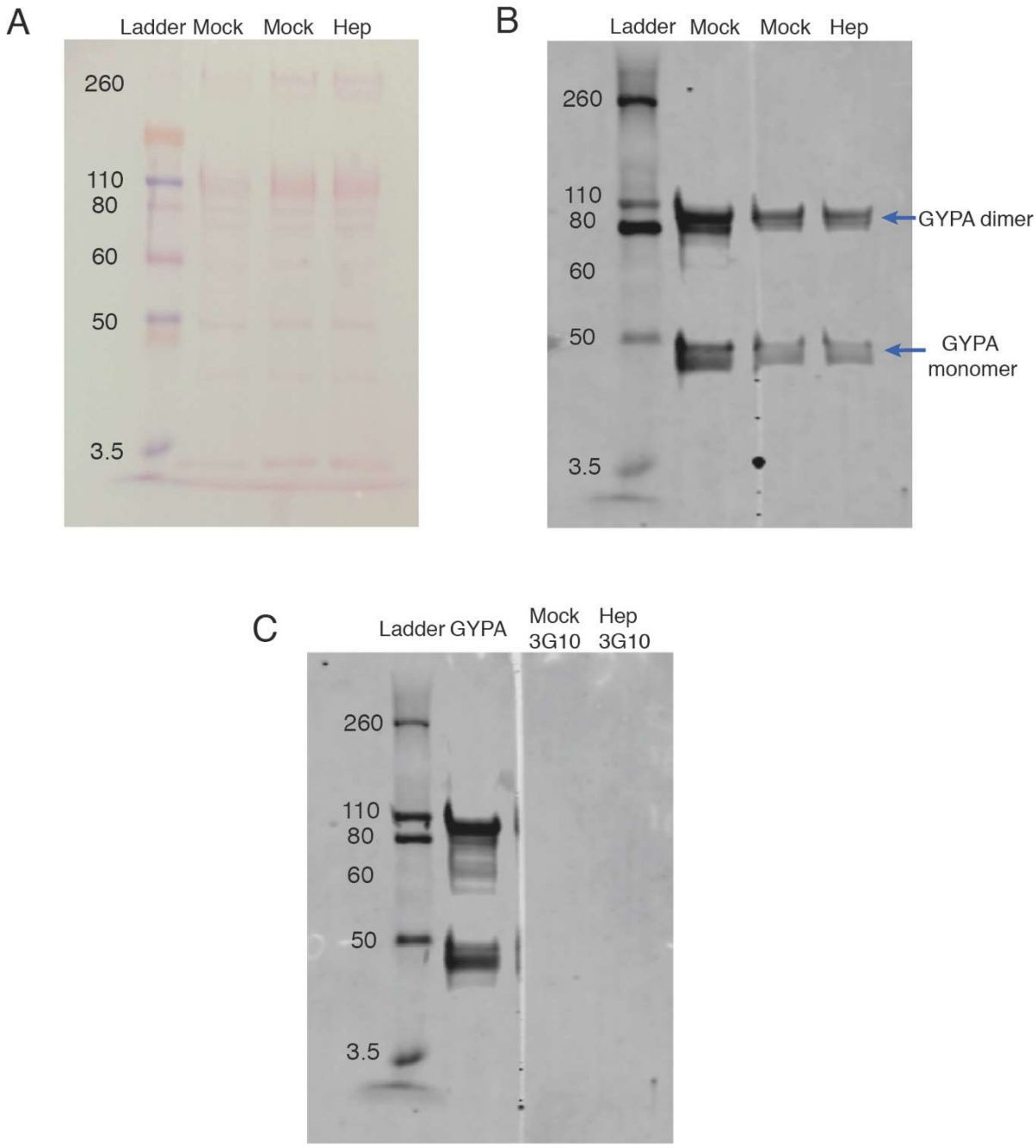


Figure 3-19 Western blot of anti-GYPA and anti-HS stub (3G10) binding for mature erythrocyte lysate. A- Ponceau staining of PVDF membrane after transfer from gel. B- Image of Western blot after stripping and reprobing with anti-GYPA antibody as a loading control to demonstrate presence of protein. C Image of original Western blot showing anti-GYPA binding in 2nd lane but no evidence of anti-HS stub binding in either mock or Heparinase III treated erythrocytes.

In order to confirm that the antibodies were capable of detecting HS on Western blotting, COS 7 cells were Heparinase III treated, lysed and blotted alongside the mature erythrocytes. Bands of the expected molecular weight (Bai et al., 1994) could be detected on the enzyme, but not mock treated COS 7 cells and no bands were seen in the mature erythrocyte lanes.

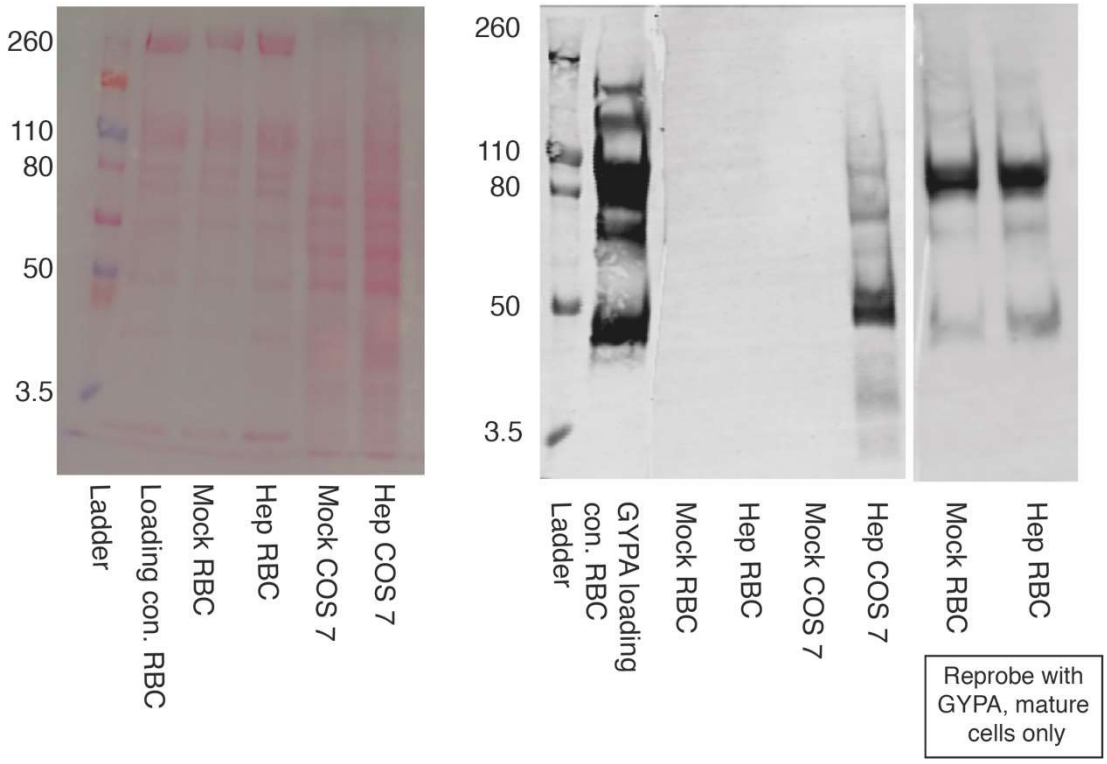


Figure 3-20 Western blot showing detection of HS stub molecule with 3G10 antibody in enzyme treated COS 7 cells only. On the LEFT, Ponceau staining of PVDF membrane after transfer from gel. On the RIGHT, western blot using GYPA antibody (loading control and reprobe on far right), or 3G10 HS stub antibody on mock or Heparinase III treated mature erythrocytes (RBC)/COS 7 cells.

This experiment was also repeated using cRBC at day 8 of culture, which express HS detectable on flow cytometry as shown above, however cell numbers were limited (approximately $5x10^6$ cells/lane). No evidence of HS could be seen however the loading control, glycophorin A (which is expressed by day 8, see figure 4.11), was also not detected suggesting the negative result was probably due to insufficient protein for detection.

3.5.7 Evaluation of published proteomic and transcriptomic data

These data described above, seem to suggest that neither HS nor CS are present in detectable quantities on mature erythrocytes, however very early erythrocyte precursors do express some HS. I therefore returned to the literature to conduct an analysis of the published data on the erythrocyte transcriptome and proteome to discover further evidence to support this hypothesis. The studies, reviews and databases used are summarised in Table 3-3.

Reference	Methodology	Output	Comments
(D'Alessandro et al., 2010)	Review and reanalysis of published red cell proteome studies to 2010	Proteome: List of 1989 individual proteins identified from mature erythrocytes	Includes membrane and cytosolic proteins
(Merryweather-Clarke et al., 2011)	RNA extraction from cultured erythroid precursors at 4 stages of maturation. Transcriptome determined using Affymetrix arrays	Transcriptome: Gene expression levels at different stages of erythroblast development	Database: Human Erythrocyte Maturation database http://cellline.molbiol.ox.ac.uk/eryth/index.html Erythroblasts were cultured from human peripheral blood mononuclear cells and sorted using flow cytometry into 4 stages*: 1. Colony-forming units (CFU-E)- 4 days in phase 2 culture 2. Pro-erythroblasts (Pro-E)- 5-6 days 3. Intermediate (Int-E)- 8-11 days 4. Late erythroblasts (Late-E)- 13-15 days
(Hegedűs et al., 2015)	Mass spectrometry on erythrocyte membranes and comparison with other published databases	Proteome: Database storing all experimentally identified mature erythrocyte proteins	Database: http://rbcc.hegelab.org/ Data included in Table 3-4 includes all proteins (not limited to membrane bound)
(Wilson et al., 2016)	Tandem Mass Tag mass spectrometry comparing proteome of matured cord or adult derived erythroid cells and autologous mature erythrocytes	Proteome: Lists of proteins detected and comparison between cells types	Data included in Table 3-4 are from either the membrane or cytosolic fractions of human RBCs (matured cRBC or autologous mature erythrocytes, supplemental table 2 from Wilson *et al.)*
(Gautier et al., 2016)	Mass spectrometry of cultured CD34+ cord blood stem cells at seven stages of maturation	Proteome: Absolute expression of 6130 proteins at different stages of erythroblast development (including copy number/cell)	Four cord donors. Cells analysed at stages: Prog1 (D8 after sorting for CD34 positivity), Prog2 (D9-11), ProE (D11-12), Baso1 (D12-13), Baso2 (D13-15), Poly (D15-17) and Ortho (D16-18), enucleated reticulocyte and pyrenocyte (extruded nucleus)

*Table 3-3 Published papers used to investigate for evidence of HS and CS expression in erythrocytes and erythroid precursors. * see also Figure 3-21*

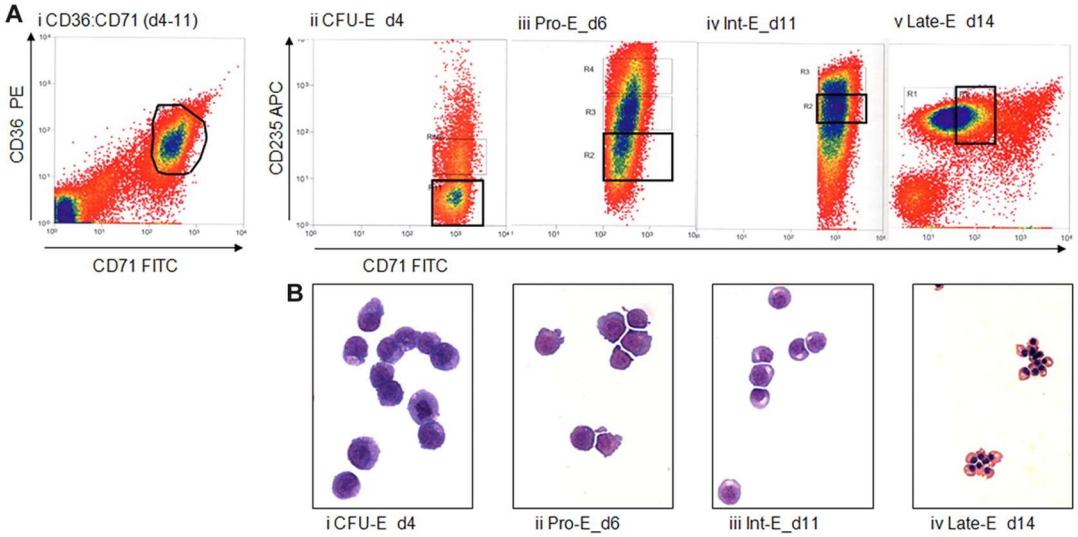


Figure 3-21 Flow cytometry and morphological criterea used to define the stages of erythroid development used by (Merryweather-Clarke et al., 2011)

Table 3-4 lists all the genes coding for enzymes involved in HS and CS biosynthesis, along with haemoglobin beta, CD71and three other potential rosetting receptors (GYPA, GYPC and CR1) which are known to be highly expressed in mature erythrocytes for comparison. The stage and maximum expression levels of each gene, derived from Merryweather-Clarke *et al*., (Merryweather-Clarke et al., 2011), are included, along with whether or not the protein product of each gene was detected from the mature erythrocytes in D'Alessandro *et al.*, the database created by Hegedus *et al.* or Wilson *et al.* (D'Alessandro et al., 2010, Hegedűs et al., 2015, Wilson et al., 2016). Of note, the gene expression levels of all the enzymes involved in GAG biosynthesis were relatively low, even in early erythroblast stages, with the maximal expression mainly at the earliest, 'Colony-forming unit' stage, corresponding to the early stages of my cRBC cultures at which HS could be detected. None of the proteins, with the exception of heparan sulfate-glucosamine 3-sulfotransferase 2, were detected in mature erythrocytes.

Table 3-4 Transcriptome of erythroblasts at different levels of maturity ('Erythroid precursors') and proteome of mature erythrocytes ('Mature erythrocytes'). Data for the maximal level of gene expression at different erythroblast stages (see Table 3-3 for abbreviations and Chapter 4: Introduction for description of the stages) are taken from Merryweather-Clarke et al., while mass spectroscopy data for the resulting proteins on mature erythrocytes is compared across three different studies/databases as shown ('Mature erythrocytes'). Black bordered cells- Molecules known to be highly expressed in mature erythrocytes/previously described rosetting receptors provided for comparison purposes. Orange border- Enzymes common to both HS and CS biosynthesis. Yellow border- HS biosynthesis. Blue border- CS synthesis. Cell shading indicates relative expression levels: Green= 'high', >1000, Orange= 'medium', 100-999, Red= 'low' 0-99.*

			Erythroid precursors		Mature erythrocytes		
Common/HS or CS/Other rosetting	**Gene name**	**Function (Stelzer et al., 2016)**	**Stage of Max expression (Merryweather-Clarke et al., 2011)**	**Max expression value (mean of 3 replicates)**	**Detected by (Wilson et al., 2016)**	**Detected (Hegedűs et al., 2015)**	**Detected by (D'Alessandro et al., 2010)**
N/A	HBB, Haemoglobin Beta	Mature RBCs	Late-E	24,762.6	Yes	Yes	Yes
N/A	TFRC, Transferrin receptor/CD71	Reticulocytes	IntE	14378.9	Yes	Yes	No
Other rosetting	CR1, Complement receptor 1	-	Cluster 7: Late-E Cluster 16: Int-E	525.40 1015.69	No	Yes	Yes
Other rosetting	GYPA, Glycophorin A	-	Int-E	4012.87	Yes	Yes	Yes
Other rosetting	GYPC, Glycophorin C	-	Late-E	1064.15	Yes	Yes	Yes
Common	XYLT1, Xylosyltransferase I	Catalyses transfer of Xylose to serine of core protein	CFU-E	23.0	Yes	No	No
Common	XYLT2, Xylosyltransferase II	Catalyses transfer of Xylose to serine of core protein	CFU-E	73.63	No	No	No
Common	B4GALT7, Xylosylprotein beta 1,4-galactosyltransferase, polypeptide 7	Attaches first Galactose in link tetrasaccharide	CFU-E	131.76	No	No	No
Common	B3GALT6, Beta-1,3-galactosyltransferase 6	Attaches second Galactose in link tetrasaccharide	CFU-E	520.61	No	No	No
Common	B3GAT3, beta-1,3-glucuronyltransferase 3	Attaches Glucaronic acid to link tetrasaccharide	CFU-E	142.40	No	No	No

HS	HSPG2, Heparan sulfate proteoglycan 2	Encodes core protein of Perlecan	Unavailable	Unavailable	No	No	No
HS	EXTL2; exostosin-like glycosyltransferase 2	Alternating addition of Glycuronic acid and N-acetylglucosamine to HS chains	CFU-E	491.87	No	No	No
HS	EXTL3; exostosin-like glycosyltransferase 3	Catalyses transfer of N-acetylglucosamine to HS chain	Similar across all stage	89.44 (CFU-E)	No	No	No
HS	EXTL1; exostosin-like glycosyltransferase 1	Glycosyltransferase, polymerises HS chain.	Late-E	46.5	No	No	No
HS	EXT1; exostosin glycosyltransferase 1	Glycosyltransferase, polymerises HS chain.	Pro-E	60.97	No	No	No
HS	EXT2; exostosin glycosyltransferase 2	Glycosyltransferase, polymerises HS chain.	CFU-E	341.40	No	No	No
HS	NDST1; N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	Catalyses both the N-deacetylation and the N-sulfation of glucosamine of heparan sulfate. Absolutely required (cf other NDST enzymes)	CFU-E (slight, non-sig upregulation in Late-E)	46.54	No	No	No
HS	NDST2; N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 2	Catalyses both the N-deacetylation and the N-sulfation of glucosamine of heparan sulfate.	Late-E	203.04	No	No	No
HS	NDST3; N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 3	Catalyses both the N-deacetylation and the N-sulfation of glucosamine of heparan sulfate.	Similar	10.59 (Late-E)	No	No	No
HS	NDST4; N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 4	Catalyses both the N-deacetylation and the N-	Similar	28.49 (Late-E)	No	No	No

		sulfation of glucosamine of heparan sulfate.					
HS	GLCE; glucuronic acid epimerase	Responsible for epimerization of D-glucuronic acid (GlcA) to L-iduronic acid (IdoA) of HS, which endows the nascent polysaccharide chain with the ability to bind growth factors and cytokines	Int-E	132.87	No	No	No
HS	HS2ST1; heparan sulfate 2-O-sulfotransferase 1	Transfers sulfate to the 2 position of the iduronic acid residue of heparan sulfate	Pro-E	374.17	No	No	No
HS	HS6ST1; heparan sulfate 6-O-sulfotransferase 1	Responsible for 6-O-sulfation of heparan sulfate	CFU-E	230.27	No	No	No
HS	HS6ST2; heparan sulfate 6-O-sulfotransferase 2	6-O-sulfation of N-acetyl-Glucosamine	Similar	8.10 (Late-E)	No	No	No
HS	HS6ST3; heparan sulfate 6-O-sulfotransferase 3	Responsible for 6-O-sulfation of heparan sulfate	Similar/Late-E	55.62	No	No	No
HS	HS3ST1; heparan sulfate-glucosamine 3-sulfotransferase 1	Catalyses the transfer of a sulfo group to position 3 of glucosamine residues in heparan. Catalyses the rate limiting step in the biosynthesis of heparan sulfate (HSact). This modification is a crucial step in the biosynthesis of anticoagulant heparan sulfate as it completes the structure of the antithrombin pentasaccharide binding site.	Late-E (/Pro-E)	59.73	No	No	No

HS	HS3ST2; heparan sulfate-glucosamine 3-sulfotransferase 2	Catalyses the transfer of a sulfo group to an N-unsubstituted glucosamine linked to a 2-O-sulfo iduronic acid unit on heparan sulfate. Catalyses the O-sulfation of glucosamine in GlcA2S-GlcNS.	Late-E	31.46	No	Yes	Yes
HS	HS3ST3B1; heparan sulfate-glucosamine 3-sulfotransferase	Catalyses the addition of sulfate groups at the 3-OH position of glucosamine in heparan sulfate Polymorphism linked with parasitaemia Atkinson *et al.* 2012	Late-E	38.73	No	No	No
HS	HS3ST3A1; heparan sulfate-glucosamine 3-sulfotransferase	Catalyses the addition of sulfate groups at the 3-OH position of glucosamine in heparan sulfate Polymorphism linked with parasitaemia Atkinson *et al.* 2012	Similar across all stages	10	No	No	No
HS	HS3ST5; heparan sulfate-glucosamine 3-sulfotransferase 5	Catalyses the transfer of a sulfo group to position 3 of glucosamine residues in heparan	Similar across all stages	18.65 (CFU-E)	No	No	No
CS	CSGALNACT1; chondroitin sulfate N-acetylgalactosaminyltransferase 1	Transfers 1,4-N-acetylgalactosamine (GalNAc) to glucuronic acid (GlcUA). Required for addition of the first GalNAc to the core tetrasaccharide linker and for elongation of chondroitin chains	IntE	145.87	No	No	No

CS	CSGALNACT2; chondroitin sulfate N-acetylgalactosaminyltransferase 2	Transfers 1,4-N-acetylgalactosamine (GalNAc) to glucuronic acid (GlcUA). Required for addition of the first GalNAc to the core tetrasaccharide linker and for elongation of chondroitin chains	IntE	236.36	No	No	No
CS	CHSY3; chondroitin sulfate synthase 3	Transfers glucuronic acid (GlcUA) from UDP-GlcUA and N-acetylgalactosamine (GalNAc) from UDP-GalNAc to the non-reducing end of the elongating chondroitin polymer. Specific activity is much reduced compared to CHSY1.	Similar	14.48	No	No	No
CS	CHSY1; chondroitin sulfate synthase 1	Transfers glucuronic acid (GlcUA) from UDP-GlcUA and N-acetylgalactosamine (GalNAc) from UDP-GalNAc to the non-reducing end of the elongating chondroitin polymer.	IntE	1015.18	No	No	No
CS	CHPF; chondroitin polymerizing factor	Transfers glucuronic acid (GlcUA) from UDP-GlcUA and N-acetylgalactosamine (GalNAc) to the non-reducing end of the elongating chondroitin polymer.	LateE	111.43	No	No	No
CS	CHPF2; chondroitin polymerizing factor 2	Transfers glucuronic acid (GlcUA) to N-acetylgalactosamine residues on the non-reducing end of the elongating chondroitin polymer. Has no N-	unavailable	unavailable	No	No	No

		acetylgalactosaminyltransferase activity					
CS	CHST11; carbohydrate (chondroitin 4) sulfotransferase 11	Catalyses the transfer of sulfate to position 4 of the N-acetylgalactosamine (GalNAc) residue of chondroitin	IntE	398.35	No	No	No
CS	CHST12; carbohydrate (chondroitin 4) sulfotransferase 12	Catalyses the transfer of sulfate to position 4 of the N-acetylgalactosamine (GalNAc) residue of chondroitin and desulfated dermatan sulfate	Late E	656.78	No	No	No
CS	CHST13; carbohydrate (chondroitin 4) sulfotransferase 13	Catalyses the transfer of sulfate to the C4 hydroxyl of beta-1,4-linked N-acetylgalactosamine (GalNAc) flanked by glucuronic acid residue in chondroitin	Late E	90.96	No	No	No
CS	CHST3; carbohydrate (chondroitin 6) sulfotransferase 3	Catalyses the transfer of sulfate to position 6 of the N-acetylgalactosamine (GalNAc) residue of chondroitin	Late E	44.61	No	No	No
CS	CHST7; carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7	Catalyses the sulfation of 6-hydroxyl group of GalNAc in chondroitin	LateE	47.71	No	No	No
CS	CHST15; carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	Transfers sulfate from 3-phosphoadenosine 5-phosphosulfate (PAPS) to the C-6 hydroxyl group of the GalNAc 4-sulfate residue of chondroitin sulfate A and forms	unavailable	unavailable	No	No	No

		chondroitin sulfate E containing GlcA-GalNAc(4,6-SO(4)) repeating units					
CS	UST; uronyl-2-sulfotransferase	Sulfotransferase that catalyses the transfer of sulfate to the position 2 of uronyl residues. Has mainly activity toward iduronyl residues in dermatan sulfate, and weaker activity toward glucuronyl residues of chondroitin sulfate. Has no activity toward desulfated heparin	Similar	18.05	No	No	Yes

*The Human Erythroblast Maturation database can be accessed via http://cellline.molbiol.ox.ac.uk/eryth/index.html

Of all the listed enzymes/proteins involved in HS and CS, only two, Heparan sulfate 2-O-sulfotransferase 1 (HS2ST1), and Basement membrane-specific Heparan sulfate proteoglycan core protein (HSPG2), were recorded in the supplemental tables of Gautier *et al.* Figure 3-22 shows a graphical representation of the copy number per cell at different stages of erythroblast maturation of these two molecules, along with glycophorin A and Band 3 for comparison (Data taken from the supplemental information of (Gautier et al., 2016)). Further details on the stages of erythroid development and images are given in the introduction to Chapter 4. Consistent with all the data presented thus far, it appears that while HS (though not CS) is present on early erythroblasts during maturation in the bone marrow, HS expression is lost before the point of enucleation and release into the peripheral circulation, and therefore cannot be detected on mature erythrocytes.

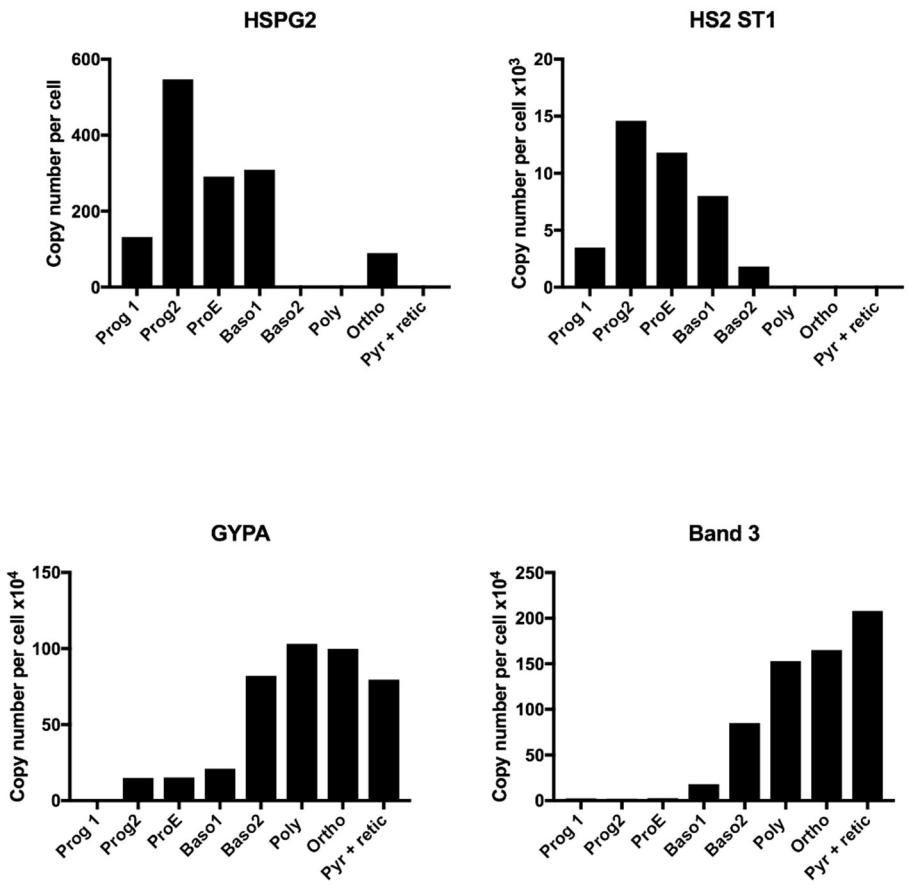


Figure 3-22 Molecular copy number per cell of Heparin sulfate proteoglycan core protein (HSPG2), Heparan sulfate 2-O-sulfotransferase 1 (HS2 ST1), glycophorin A (GYPA) and Band 3. Note differing scales on y-axis. Data on copy number taken from supplemental information of (Gautier et al., 2016).

3.6 Discussion

Heparin sulfate has long been considered an important rosetting receptor despite much of the supporting evidence failing to convincingly demonstrate a specific interaction between the parasitized erythrocyte and HS native to the host erythrocyte cell membrane. The aim of my work was to further explore HS and CS as potential rosetting receptors using carefully validated methods designed to identify whether these molecules are actually expressed on circulating uninfected erythrocytes and if removing them had any effect on the rosetting ability of a range of parasites.

3.6.1 Heparan sulfate and chondroitin sulfate are not specific rosetting receptors

Initially, I used an enzymatic approach to remove both HS/CS from the mature erythrocytes in the hope that this would provide evidence of a specific interaction between the infected cells and HS/CS present on the uninfected erythrocyte membrane. However, no effect on rosetting was seen, and, interestingly, removal of HS also had no effect on the ability of heparin to disrupt rosettes, suggesting that different mechanisms were at play. When the removal of HS/CS also made no difference to binding of rPfEMP1 domains, it seemed prudent to question the basic assumption that either of these molecules were present on mature erythrocytes. Consistent with my results thus far, I was unable to identify any evidence of HS or CS on mature erythrocytes. While this could have initially represented a problem with my detection technique, confidence in my methods was restored by the identification of HS on early erythrocyte precursors.

To a certain extent, these findings contrast with the existing published literature. As a direct comparison, Barragan *et al*. (Barragan et al., 1999) tested two parasite lines, FCR3S1 (analogous to ITvar60) and TM284, using the same enzymatic technique and found substantial reduction in rosetting

frequency for both. However, there are some potential issues with this paper; the results are presented as percentage of control and no statistical analysis comparing the treated and control samples are given. This can be misleading, for example if the change in rosette frequency is from 10% to 5%, this can be presented as a 50% reduction in rosette frequency but variation of level is commonly seen even within the same culture. The additional experiments in which only the uninfected cells are treated are also problematic; enzyme-treated, uninfected cells are labelled then mixed with untreated, parasite cultures (containing both infected and uninfected cells which have not been mock treated or labelled). The rosetting ability of the enzyme-treated uninfected cells and the uninfected cells already in the culture are then compared. No data are given to demonstrate that labelling does not affect rosetting, or that pre-existing rosettes were fully disrupted before mixing with treated cells (the 'mechanical disruption' of rosettes described in the paper is not effective in my experience). It is therefore arguably not an appropriate comparison and any decrease in rosetting of the enzyme treated cells could simply be due to the labelling or a non-specific effect of enzyme treatment.

As discussed in the introduction, much of the evidence suggesting that HS is a rosetting receptor, relies on the use of heparin or soluble HS to disrupt rosettes. My data support the finding that heparin does disrupt rosettes across a range of parasite strains. However, the results of my enzyme experiments, and the lack of effect of enzyme treatment on PfEMP1 binding to uninfected erythrocytes, have led me to conclude that the mechanism of disruption is not blocking of host HS on uninfected erythrocytes. Rather, it seems likely that either the highly negatively charged heparin molecules are able to interrupt rosetting in a non-specific, charge related fashion, or that heparin is binding to PfEMP1 thus blocking rosetting from the parasite side, rather than host. There is some evidence to support both these hypotheses. Barragan *et al.* found that sulfation, and by extension charge, was important for rosette disruption ability with desulfated heparin having minimal effect on

rosetting (Barragan et al., 2000a). The binding of Duffy-like-binding domains from the VAR2CSA protein to Chondroitin sulfate can also be disrupted by relatively low concentrations of soluble HS, which Resende *et al.* concluded was evidence of the non-specific, charge related nature of this interaction (Resende et al., 2009). PfEMP1 has been showed to bind HS as seen in the glycan array, which reignited our interested in the GAGs as rosetting receptors, and multiple other studies (Barragan et al., 2000a, Heddini et al., 2001, Juillerat et al., 2011, Adams et al., 2014). It seems quite plausible that the disruption of rosettes by heparin/soluble HS is in fact due to binding of PfEMP1, as suggested by Juillerat *et al.* (Juillerat et al., 2011), rather than blocking of host HS on the uninfected erythrocytes. Heparin binding to parasite derived molecules could also explain the results of several papers which propose heparan sulfate as a potential erythrocyte invasion receptor (Boyle et al., 2010, Kobayashi et al., 2010).

3.6.2 Heparan sulfate and chondroitin sulfate are not present on mature erythrocytes: a comparison with the literature

These data and hypotheses are all consistent with the second major conclusion from this work; that it is unlikely that either HS or CS are present in detectable, and possibly by extension clinically relevant, quantities on mature erythrocytes. As alluded to previously, this is at odds with the single paper which suggests that HS can be found on mature erythrocytes (Vogt et al., 2004). When replicating the experiments described in Vogt *et al*., I identified a number of potential issues which could lead to a different interpretation of their results. In the first instance, the methods, namely 37^{o}C incubation times and a double layer secondary and tertiary antibody technique, may not promote *specific* binding. However the appropriate comparison of the mock and enzyme treated cells should overcome this issue. When comparing mock and enzyme treated cells, I found a significant amount of background fluorescence, along with multiple dots, which could appear to be clusters of HS, except these areas were seen equally in both

control and treated samples (Figure 3-13). The single cell depicted in Vogt *et al.* shows similarly high levels of background fluorescence and bright dots, however the background of the control samples is significantly darker, perhaps suggesting different microscopy settings. Figure 3-23 compares the two image sets. Similarly, the flow cytometry plots depicted in Vogt *et al*. appear to show a large number of cells on the y-axis suggesting different voltage settings to those used for my analysis.

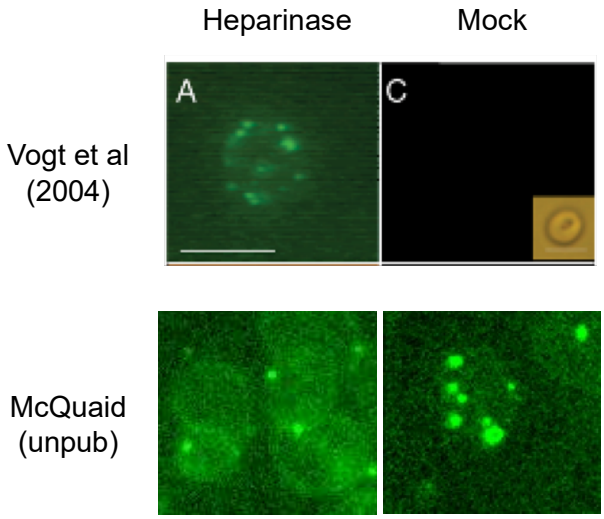


Figure 3-23 Comparison of images from Vogt et al. (2004) and single cells taken from Figure 3-13 showing mock and enzyme treated erythrocytes incubated with 3G10 primary antibody followed by secondary and tertiary antibodies. Colour has been added to the images from this thesis with Image J but no other image manipulation has taken place.

It is important to note that Vogt *et al.* did conduct additional experiments involving purification of O-glycans which they conclude came from the mature erythrocytes and used a different method of blotting red cell membranes. However these methods involved lysing the cells therefore it is not possible to determine whether the O-glycans detected in these experiments was expressed on the cell surface or just intracellularly. They also separated fully mature erythrocytes from reticulocytes and report no differences in the expression of HS between these cell types. This differs from my findings that HS is expressed at very early erythrocyte precursor

stages but is lost with maturity, however the literature review presented in section 3.5.7 is more in support of my data. Though there are some variations between the studies, broadly speaking, there is some relatively low-level gene transcription of HS and CS biosynthesis genes at a very early stage of erythroid development, but very few of the corresponding proteins are expressed at later stages. Despite this, it is important to bear in mind that it is impossible to prove that something does not exist, and I cannot rule out the possibility that HS (or indeed CS) is expressed in very low levels on mature erythrocytes.

3.6.3 Limitations of this work

The other limitations of this work must also be acknowledged. All the experiments, and parasite culturing, have been carried out using blood from Scottish blood donors. Though the ethnicity of these donors is unknown, it is reasonable to assume, given the demographics of the Scottish population, that the majority of these would be of Caucasian origin, and all would be over the age of 17 years. Arguably, the erythrocytes of particular interest concerning severe malaria are those of the groups most affected by severe disease, namely African children. It is possible that children in Africa do express high levels of HS of their erythrocytes (either due to their ethnicity or age) and this is why they are more susceptible to severe disease. However, this seems unlikely; if high levels of HS do promote severe disease, one might expect this trait to be selected out of the African population and it would be unusual for a trait which predisposes to severe malaria to be present in the Africans but not Caucasians (in contrast, for example, with sickle cell trait).

The use of enzymes and antibodies to stub molecules may seem a somewhat convoluted and indirect way to attempt to detect HS and CS. However, as discussed in the introduction, HS and CS are both inherently highly variable molecules and antibodies to the whole molecule of one

variant may not bind well to another. Using the common stub antibodies allows for the vast range of HS/CS types, and the superior detection compared to antibodies directed against the whole molecule can be seen in Figure 3-8. Nevertheless, while the stub antibodies can demonstrate that the enzymes are functional, they cannot determine to what degree the HS or CS is removed from the cell surface. I cannot rule out that a small quantity remains even after enzyme treatment and that HS/CS are potent enough rosetting receptors that only a few molecules are sufficient to bind to infected cells.

There are also a number of technical and methodological issues which could be optimised. While the concentration of Heparinase III was consistent throughout in both the COS cell validation and erythrocyte experiments, the cell numbers were different (for example $8x10^8$ erythrocytes versus $1.5x10^6$ COS cells in the Western blot assays). Ideally these cell numbers would be better matched, however one must also bear in mind differences in cell size. While the COS cell results demonstrate that the enzyme does function as expected under similar conditions to those used for the erythrocyte experiments, this does not absolutely prove that Heparianse III is capable of removing HS specifically from mature erythrocytes in large cell numbers. Additionally, glycophorin A was perhaps not the most appropriate positive control to use for Western blot experiments as it is one of the most abundantly expressed molecules on the erythrocyte surface. A protein with a lower copy number, for example CR1, may have been a better choice to confirm that the experimental methods were capable to detecting molecules with lower expression levels.

3.6.4 Conclusions, clinical context and the way forward

Nevertheless, the results of the experiments contained within this chapter have led me to believe that HS is expressed in the very early stages of erythroid cell development but is lost before the reticulocytes are released

into the peripheral circulation. I am unable to discover any experiments that HS is an important, specific rosetting receptor on uninfected host erythrocytes. This also has important implications outside the rosetting field; HS has been proposed as an erythrocyte invasion receptor and my data suggest that further research into this receptor in an invasion context may not be particularly fruitful. The data on CS are new and, though negative, do present useful data to add to the literature. However, this is not to say that heparin, heparan sulfate and its derivatives are useless in the fight against severe malaria. The mechanism may not be specific, but heparin does disrupt rosettes very effectively. In itself, heparin is not a useful therapy due to its anticoagulant effects (Smitskamp and Wolthuis, 1971, WHO, 1986), but the low anticoagulant heparin derivative, Sevuparin, is already undergoing clinical trials as an adjuvant therapy for severe malaria (Leitgeb et al., 2017, Saiwaew et al., 2017). *In vitro* clinical isolates cultured from uncomplicated adult malaria cases in Thailand had a median rosette disruption effect of 40% at therapeutic concentrations of Sevuparin (estimated to be around 100μg/ml) and adherence to human dermal endothelial cells was also reduced (Saiwaew et al., 2017). A small clinical trial of Sevuparin in Thai patients with uncomplicated malaria also showed apparent desequestration of mature parasites with no safety issues (Leitgeb et al., 2017). This is particularly interesting as it provides the first clinical data suggesting that rosette disruption may be a safe therapeutic target. It appears that it's not yet time to ignore the potential anti-rosetting effects of heparin and its analogues, however this makes elucidating the precise mechanism even more important.

One of the important strengths of my work is the use of a range of actual parasites and erythrocytes which therefore present the molecules of interest, both host and parasite derived, in as natural a way as possible *in vitro*. The glycan screening array is an example of the perils of using recombinant proteins on artificial matrixes which may not represent the actual molecules present on mature erythrocytes. However, the "block" and "chop" techniques

used still leave much to desired in terms of specificity and screening potential. This highlights the need for new approaches to investigating host rosetting receptors. A more elegant solution could be the use of knockout erythrocytes which lack the receptor of interest. These cells could be tested on multiple laboratory and clinical strains and would have the benefit of being otherwise ‘normal’ erythrocytes in terms of shape and surface molecules. The remainder of my thesis is therefore dedicated to developing a new model for investigating host erythrocytes rosetting receptors using knockout erythrocytes to elucidate the true mechanisms of rosetting in severe malaria.

4. CHAPTER IV: USING CULTURED HAEMATOPOIETIC STEM CELLS TO INVESTIGATE POTENTIAL ROSETTING RECEPTORS AND IDENTIFY A NOVEL ROSETTING RECEPTOR

4.1 Abstract

The ability to generate 'designer' erythrocytes would be an extremely useful tool in determining the essential host-related mechanisms of rosetting. Experiments could be performed using well-characterised, knockdown/out erythrocytes, which express the remainder of the cell surface receptor complement as close to *in vivo* as possible. Such cells could be tested with multiple parasite lines to untangle which host receptors have an essential or accessory function for rosetting. Generating such cells is challenging as genetic manipulation must take place at the immature, erythroid precursor stage when the cell still contains a nucleus. This chapter deals with two sources of erythroid precursors; pilot experiments using induced pluripotent stem cell erythroid cultures (iPSC), and CD34+ haematopoietic stem cells derived from adult bone marrow (cRBC). Cells originating from iPSC were immature, heterogeneous and displayed an unusual adherence phenotype, however cRBC proved more promising. cRBC were characterised at multiple stages of maturation in terms of morphology, receptor expression and rosetting ability. Once matured, cRBC demonstrated excellent enucleation and showed a reticulocyte-like receptor profile indicating these cells were a good approximation of peripheral erythrocytes. The binding of recombinant rosetting PfEMP1 domains to cRBC was equal to that of mature cells, however rosetting experiments showed an unexpected result. Two parasite lines failed to rosette with cRBC, prompting a deeper investigation into the subtle differences in receptor expression. This led to the identification of Band 3 as a potentially important rosetting receptor, and further experiments showed that antibodies to the Wright^{b} antigen, carried on Band 3, had a remarkable ability to disrupt rosettes across all tested parasite strains. Attempts to generate knockdown cRBC had limited success and demonstrated the need for an immortalized erythroid line to fully realise the potential of this "knock" approach.

4.2 Introduction

4.2.1 A new method for investigating rosetting receptors; the "knock" approach

In Chapter 3, I discussed the importance of using 'real' infected and uninfected erythrocytes, as opposed to relying on recombinant proteins and other cell types. I also highlighted the challenge of dissecting whether a rosette disrupting molecule, such as heparin, is interfering with receptors on the parasitized or uninfected cells. In order to fully understand the mechanisms of rosetting, this is an importance distinction to make. I therefore decided to explore a different approach to the "block" and "chop" techniques used in the previous chapter, namely a "knock" methodology; the production of erythrocytes with reduced or absent expression of various receptors of interest. For the purposes of this thesis, I will refer to cells which have been manipulated using RNAi methods as knock-down cells, and cells which had had a permanent genome change (such as those generated using CRIPSR/Cas9 techniques) as knockouts. The advantage of using knockdown/out erythrocytes, is that, in theory (and depending on the function of the molecule), they will maintain the rest of the erythrocyte surface receptor complement, presented in a way as close to *in vivo* as possible. This allows the necessity of the knockdown/out receptor to be determined and the potential of the parasite to utilize accessory receptors to be explored. Knockdown cells have been used in such experiments to demonstrate that glycophorin A is an important invasion receptor for some *P. falciparum* strains (Bei et al., 2010) and in an exciting screening study which identified CD55 as a new, essential invasion molecule (Egan et al., 2015). The generation of knockdown erythrocytes has, in fact, already been used as a tool in the rosetting field (Lee et al., 2014, Niang et al., 2014). Lee *et al.* transduced CD34+ umbilical cord haemopoietic stem with short hairpin RNA targeted against glycophorin C (GYPC) to knockdown GYPC expression, sorted the cells based on GFP expression (indicating successful

transduction) and cultured the cells to maturity. The matured cells were then tested for rosetting ability with *P. vivax* isolates (Lee et al., 2014). They reported significantly reduced rosetting of the knockdown cells compared to control cells (the non-GFP expressing cells cultured to maturity in parallel) for the *P. vivax* isolates tested, but the experiments were not repeated for *P. falciparum*. Niang *et al.* (2014), transduced umbilical cord cells in a similar manner, and compared rosetting of the knockdown cells to controls using a single *P. falciparum* line, 5A, a clone of 3D7 (Niang et al., 2014). Again, they found a significant reduction in rosetting.

Both these studies are limited in their sole focus on GYPC and that only one laboratory adapted *P. falciparum* strain was used; Niang *et al.* only assessed 5A, and Lee *et al.* used *P. vivax* only. However, they demonstrate the proof of principle that knockdown erythrocytes can be used for rosetting assays.

4.2.2 Generating knockdown cells

4.2.2.1 Naturally occurring polymorphisms

Instead of generating artificial knockdown cells, a logical solution could be sourcing cells with naturally occurring polymorphisms leading to a decrease in receptor expression. In fact, Rowe *et al.* (1997) previously tested 23 erythrocyte variants (see Table 4-1), though not all these polymorphisms lead to a decrease or null receptor expression.

Table 4-1 Rare blood group variants tested by (Rowe et al., 1997) for rosetting phenotype

Variant tested	Antigen	Polymorph-isms tested	Effect on rosetting	Comments
Knops null	CR1	null	Yes, reduced	<100 CR1 molecules/ erythrocytes
Kell null	Kell	K_o	No	Express no Kell antigens
Bombay	H antigen	Bombay	No	No H, A or B antigen
Rh null	Rhesus	Null	No	No Rh antigens (including D, E etc.)
LW	ICAM4	a-b-	No	
McLeod	XK	McLeod (lack of XK gene)	No	Absence of XK leads to weak Kell expression and neuromuscular problems
Kidd null	JK antigen	Jk-	No	Rare in most populations, 0.9% of Polynesians
Duffy	Duffy	a-b-	No	Duffy negativity is rare in Caucasians but present in 68% of Black populations (invasion receptor for *P vivax*)
Gerbich	GYPC and GYPD	-1,-2,-3	No	Leads to elliptocytosis, not a true null phenotype (cf Leach phenotype). Common in Papua New Guinea (Patel et al., 2001)
Lutheran	Lutheran	a-b-	No	
Gregory	Gregory	a-	No	
JMH null	Semaphorin 7A	null	No	
M^kM^k	GYPA and GYPB		No	Null for GYPA and GYPB with diminished Band 3 function
Scianna	Erythrocyte membrane associated protein, ERMAP	-1,-2	No	

P	P antigens	Tja-, now referred to as p	No	
Colton	Aquaporin 1	a-b-	No	
Adult i	I	Adult i	No	i antigen is present normally until 18 months of age when it converts to I (Yu et al., 2001)
Chido negative	Complement C4	null	No	Not an intrinsic erythrocyte protein, adsorbed from the plasma
Lewis	Lewis	a-b-	No	Also adsorbed from plasma. The tested polymorphism is more common in Black, African and Indian populations.
Xg negative	Xg(a) and CD99	null	No	Gene located on X-chromosome
Diego	Band 3	b-	No	Diego^{a} is seem most commonly in South American (36%) and Asians of Mongoloid origin (5-15%)
Lan negative	Lan, carried on ABCB6	null	No	Null is rare but commoner in black South Africans (1:1500) (Peyrard, 2013)
Vel negative	Vel/small integral membrane protein 1	null	No	Null is extremely rare but clinically significant for transfusion reactions

Details on the antigens and information in the comments are taken from (Rowe et al., 1997), (Dean, 2005) and (Cooling, 2015)

While this approach led to the identification of CR1 as a rosetting receptor (Rowe et al., 1997), sourcing these very rare erythrocytes can be difficult and obtaining a continuous supply for multiple experiments is extremely challenging. The receptor expression can vary between individuals and for some receptors, for example Band 3, the null phenotype is not normally compatible with life (Ribeiro et al., 2000).

I was therefore keen to develop techniques which would allow me to 'design' my own knockdown/out erythrocytes and, potentially in the future, to make multiple knockouts in a single cell. However, mature peripheral erythrocytes are not amenable to genetic manipulation given their enucleated state. Like those conducting the knockdown studies described above, I needed to find a source of nucleated, erythroid precursors which I could manipulate, then differentiate into cells as close to mature, enucleated, peripheral erythrocytes as possible.

4.2.2.2 Sources of erythroid precursors

On entering the world of laboratory generated erythrocytes, I had the advantage of many years of research undertaken by others in the field of blood transfusion. A great deal of work has gone on, and continues, into reaching the holy grail of culturing large quantities of enucleated erythrocytes which could be used for blood transfusion purposes (Anstee et al., 2012). The major sources of erythroid precursors used in these transfusion-related studies have been:

1. Haematopoietic stem cells (HSC) derived from adults (either from bone marrow samples or obtained from the peripheral circulation with or without stimulation with Growth colony stimulating factor [G-CSF]).
2. HSC derived from the umbilical cord of newborn infants.
3. Human induced pluripotent stem cells (iPSC).

4. Human embryonic stem cells (ethical consideration may limit the use of such cells).

In addition, in recent years exciting new developments have been published regarding immortalized human erythroid lines (Kurita et al., 2013, Trakarnsanga et al., 2017) and these will be dealt with in detail in Chapter 5.

After initial pilot work with human iPSC, I decided to use commercially available HSC derived from adult bone marrow donors. However, I was also able to test one sample of umbilical cord derived HSC which were a kind gift from Professor Lesley Forrester. Further details on the characteristics of HSC and iPSC are described below.

4.2.2.2.1 Haematopoietic stem cells

CD34+ haematopoietic stem cells (HSC) are primary cells which have developed from pluripotent haematopoietic stem cells and, under the correct conditions, have the potential to expand and differentiate into mature, enucleated erythrocytes (Figure 4-1, adapted from (Attar, 2014), (Alberts et al., 2014, Nandakumar et al., 2016).

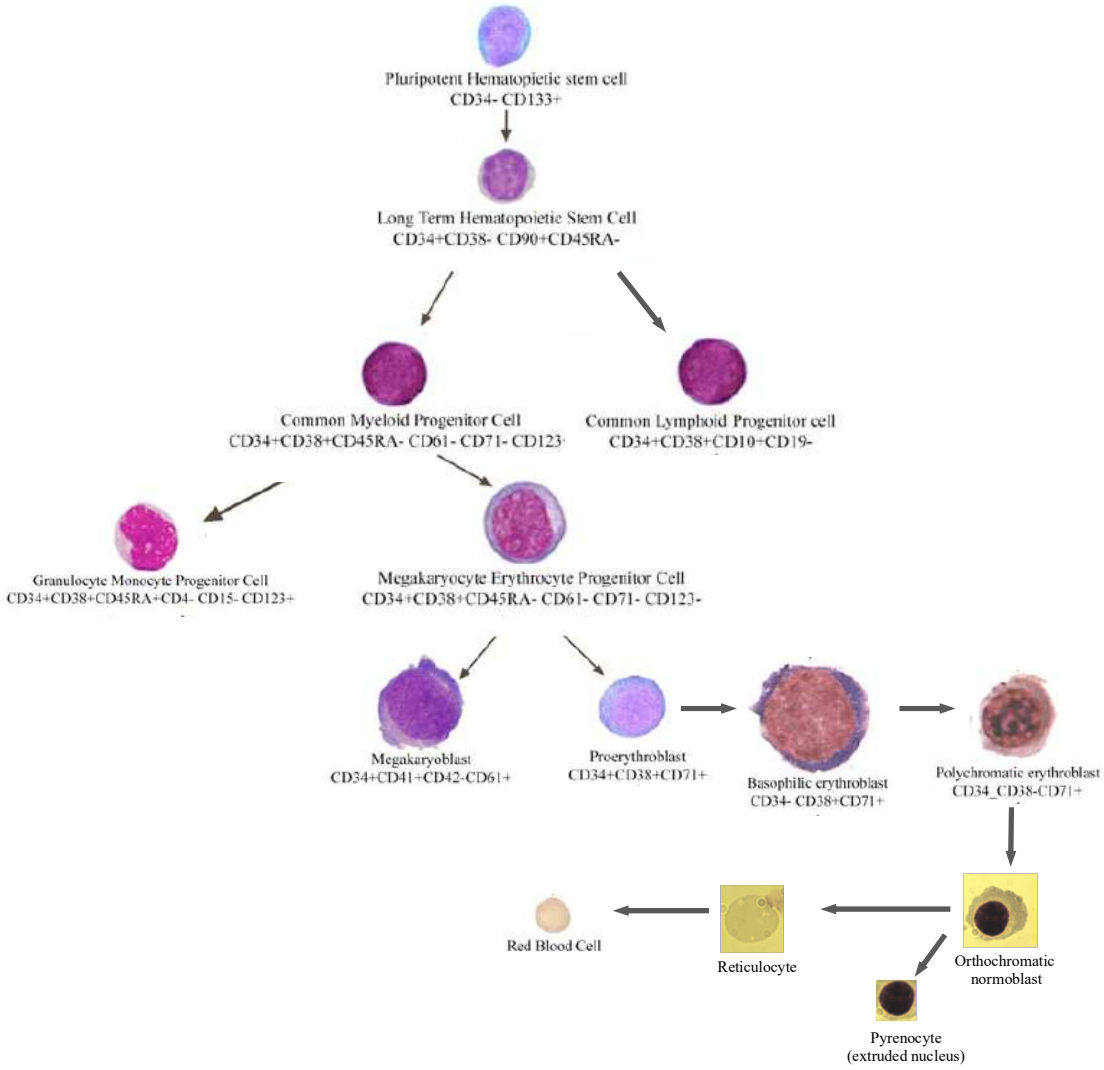


Figure 4-1 Development of haematopoietic stem cells in the bone marrow. Adapted from (Attar, 2014), the maturation stages of the other cells, including monocytes and neutrophils from the granulocyte progenitor cells, platelets from the megakaryocytes and B cells from the common lymphoid progenitors have been removed for clarity. The orthochromatic normoblast, pyrenocyte and reticulocyte images are from my own cRBC cultures.

In one of the key final stages of development, the immature erythroid precursors lose their nuclei to form reticulocytes. Enucleation occurs between the orthochromatic erythroblast and reticulocyte states and the extruded nucleus is referred to as a pyrenocyte (Figure 4-1, (Nandakumar et al., 2016)). This enucleation step makes the erythrocyte significantly more flexible allowing it to pass through microvessels much smaller that the cells itself (Guzniczak et al., 2017). Reticulocytes still contain RNA, are more metabolically active, have larger volume, and have not yet formed the classic

biconcave disc of mature erythrocytes (Piva et al., 2015). They remain in the bone marrow for around three days before release into the peripheral circulation where they reach full maturity, probably through modification by the spleen, in around 24 hours (Anstee et al., 2012, Piva et al., 2015). The presence of numerous nucleated erythrocytes in the peripheral circulation is suggestive of pathology (Constantino and Cogionis, 2000).

In postnatal mammals*, in vivo*, erythropoiesis occurs in the bone marrow (Nandakumar et al., 2016), however it is possible to culture HSC *in vitro* into relatively mature, reticulocyte-like cells (Anstee et al., 2012). These cultured erythrocytes (cRBC) have a moderately high level of enucleation (68% and above reported by (Giarratana et al., 2011)), but tend to be slightly larger, less deformable, and express a more immature repertoire of proteins on the cell surface (Giarratana et al., 2005, Giarratana et al., 2011, Anstee et al., 2012, Hu et al., 2013, Wilson et al., 2016, Guzniczak et al., 2017). Figure Figure 4-2, taken from (Guzniczak et al., 2017), demonstrates how the mechanical properties of the cultured HSC develop over the three week culture period.

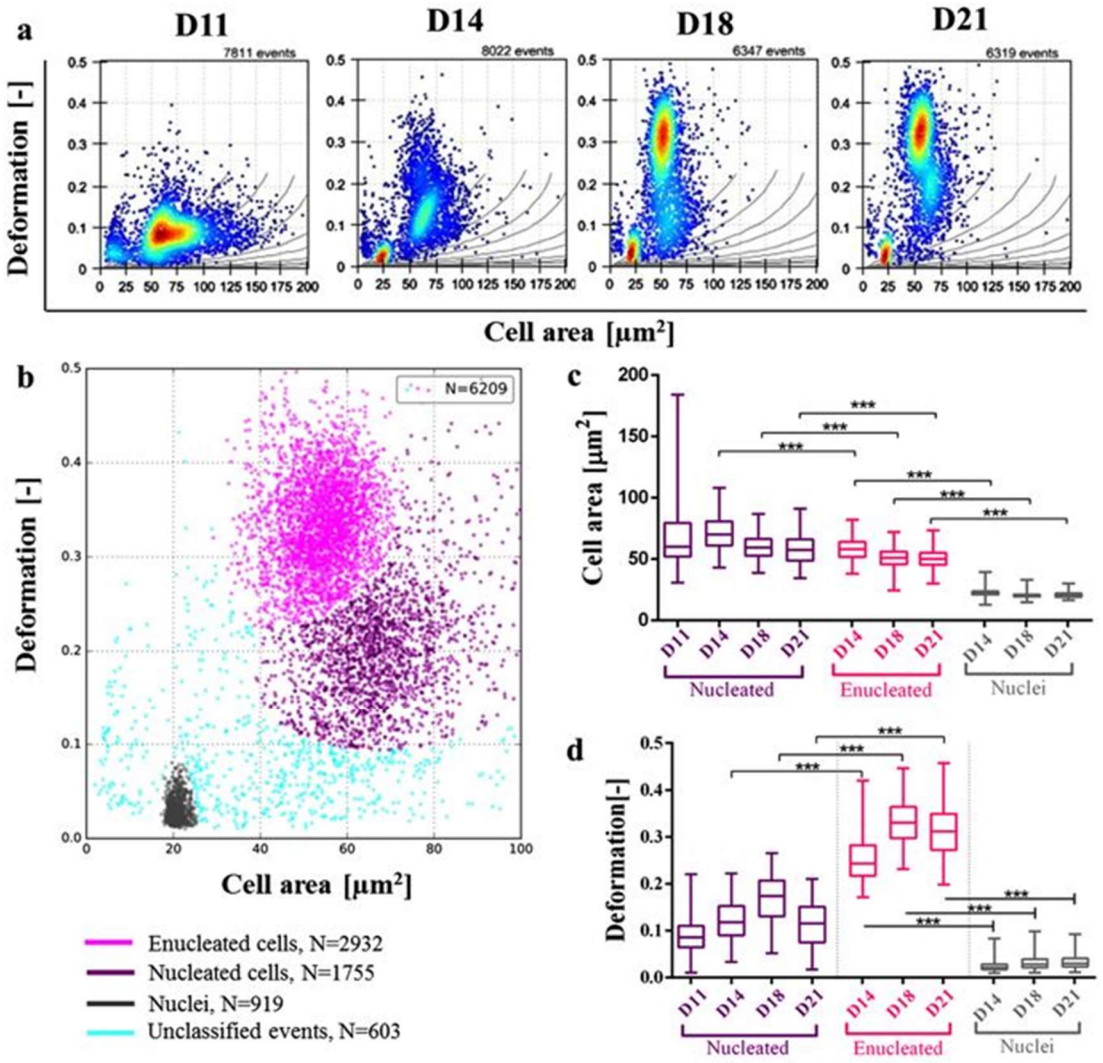


Figure 4-2 (Guzniczak et al., 2017) cultured CD34+ HSC in a similar manner to that described in section 2.2 then assessed the deformability of cell using a Real Time Deformability Cytometer. A shows the change in cell area and deformation index at 4 time points. B shows the range of cell phenotypes present at D18 of culture, coloured coded based on nucleation status and C + D show the cell size/deformability of nucleated, enucleated and the extruded nuclei at different time points. Figure taken directly from (Guzniczak et al., 2017)

Giarratana *et al.* analysed the haemoglobin (Hb) content and oxygen binding of cRBC derived initially from leukapheresis of an adult stimulated with G-CSF (Giarratana et al., 2011). They found around 10% of the Hb content was foetal Hb, which they attributed to a stress response to the various growth factors in the culture medium, and the remainder was normal adult HbA with an oxygen binding capacity similar to that of normal peripheral erythrocytes (Giarratana et al., 2011). In contrast, cRBC derived from umbilical cord stem cells contain predominantly fetal Hb, though the surface receptor profile is

similar to that of adult derived cells and they do have the advantage of a higher proliferative capacity (Wilson et al., 2016).

In early systems, CD34+ HSC required co-culturing with murine MS-5 stroma cells, or other mesenchymal cells, for support and to promote differentiation (Giarratana et al., 2005), however the methods have since been simplified and cells can now be matured independently (Giarratana et al., 2011). Autologous HSC have even been matured *ex-vivo* then injected back into the original human donor where the survival of the cRBC at 26 days was between 41 and 63% (Giarratana et al., 2011).

As discussed above, cRBC have been successfully genetically manipulated to produce knockdown cells using both RNA interference (for example, (Bei et al., 2010, Niang et al., 2014, Lee et al., 2014, Egan et al., 2015) and knockouts using CRISPR/cas9 methods (Mandal et al., 2014, Hu, 2016, Yu et al., 2016). However, cRBC do have a number of disadvantages which can impose limitations on their usefulness. The main problem is that cRBC are not immortalized cells and have limited proliferation capacity (Zeuner et al., 2012, Hu et al., 2013). This can pose a particular technical challenge for experiments which require transduction and selection of modified cells. Culturing is also very expensive and somewhat time-consuming. I was therefore keen to also explore other potential sources of erythroid precursors, namely induced pluripotent stem cells.

4.2.2.2.2 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSC) are an attractive potential source of erythroid cells due to their immortalized nature. Takahashi *et al.* first described the ‘reprogramming’ of human adult dermal fibroblasts into cells capable of differentiating into any cell type, by transducing the fibroblasts with the transcription factors Oct3/4, Sox2, Klf4 and c-Myc (Takahashi et al., 2007). Erythroid cells have been generated from iPSC ((Lapillonne et al.,

2010) and reviewed in (Easterbrook et al., 2016)), however culturing and differentiation is complex, the cells generated from this source have very poor enucleation (4% in (Lapillonne et al., 2010)), and express mainly foetal haemoglobin (Lapillonne et al., 2010, Easterbrook et al., 2016, Olivier et al., 2016, Yang et al., 2017, Ferreira et al., 2018). Nevertheless, I was fortunate enough to have access to samples of erythroid cells derived from iPSC from Professor Forrester's laboratory based in the Scottish Centre for Regenerative Medicine. I therefore decided to use these cells in pilot rosetting experiments, with a view to investigating iPSC as a potential tool to develop null erythrocytes if these pilot experiments showed promise.

4.2.2.3 Knockdown using RNA interference

Once a suitable source of erythroid precursors has been identified, the next stage is to generate the knockout or knockdown cells. The genetic manipulation tool of the moment is CRIPSR/Cas9 (Jinek et al., 2012, Pyzocha et al., 2014), however, though this technology has been used successfully with HSC (Mandal et al., 2014, Hu, 2016, Yu et al., 2016), the efficiency *in vitro* was often poor (Yu et al., 2016). Given the limited life-span of the cRBCs, generating a useable knockout population is technically very difficult, therefore I decided initially to focus on the tried and tested short hairpin RNA (shRNA) interference methods used to generate knockdown cells in the studies described above.

RNA interference, specifically the use of shRNA, is a gene expression silencing method in which messenger RNAs (mRNA) are targeted by small/short interfering RNA (siRNA) and cleaved by proteins in the RNA-induced silencing complex (RISC) (Lord et al., 2009). A schematic of the process is showed in Figure 4-3. The shRNA DNA is introduced into the cell by transduction with lentiviral particles, where it is then transcribed into shRNA and exported from the nucleus (Lord et al., 2009, O'Keefe, 2013). The shRNA is converted into double stranded siRNA consisting of around

19-25 base pairs by the enzyme Dicer, and the siRNA are then incorporated into the RISC. The RISC then uses the siRNA to identify target mRNA through complementary base pairing, and cleaves it thus silencing protein expression (Lord et al., 2009, O'Keefe, 2013).

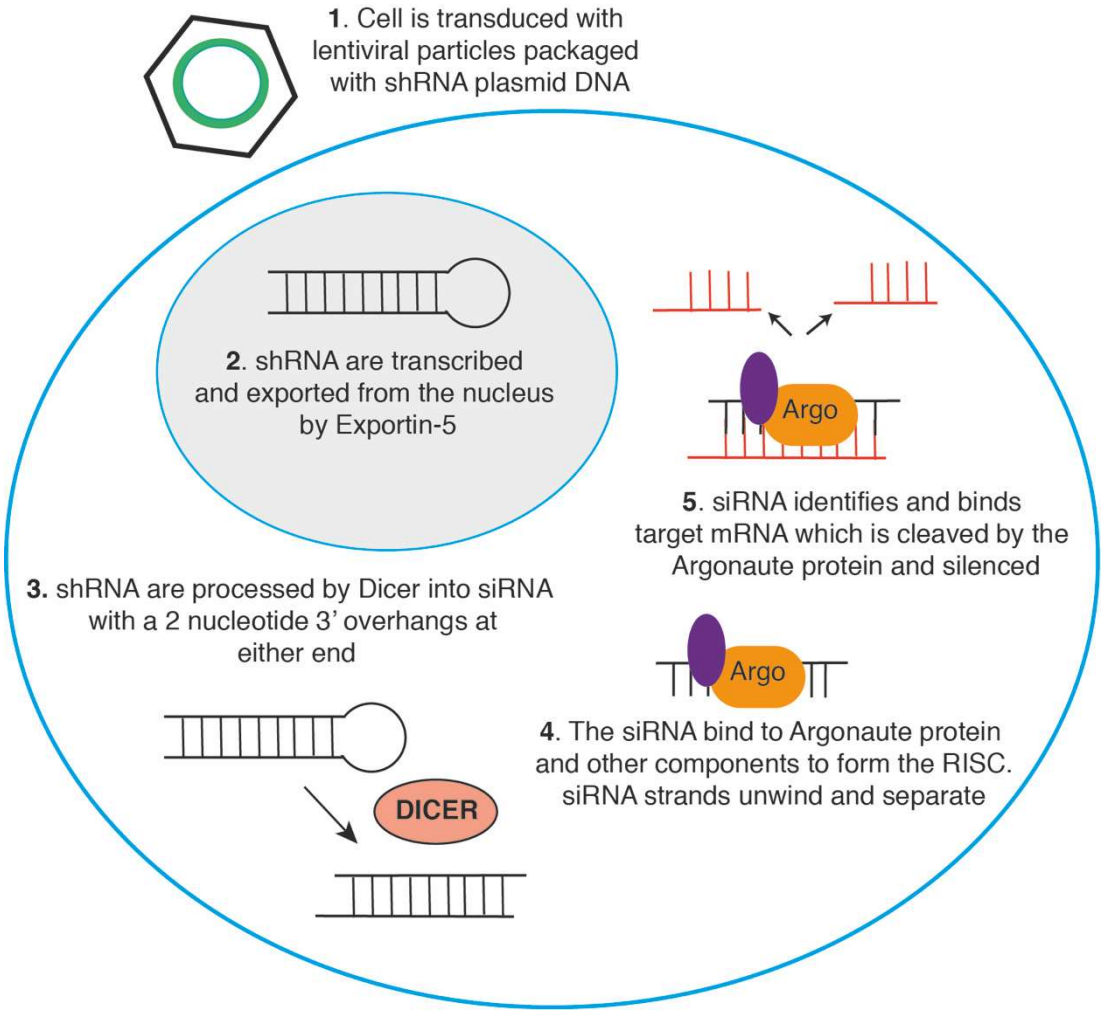


Figure 4-3 Schematic of a cell undergoing mRNA silencing using shRNA lentiviral particles. The grey shaded area represents the cell nucleus. shRNA= short hairpin RNA, siRNA = small interfering RNA, RISC = RNA inducible silencing complex, mRNA= messenger RNA

In this way, I hoped to generate a panel of different cRBCs, each with different receptor knockdowns to help distinguish which of the currently proposed receptors are essential for rosetting across multiple parasite strains.

4.3 Aims

The aim of this chapter was to investigate the utility of iPSC and cRBC as tools for determining essential host rosetting receptors. Specifically, I wanted to characterize the matured cRBC in terms of morphology, receptor profile and rosetting phenotype, and explore the possibility of generating knockdown cells for GYPA, GYPC and CR1.

4.4 Methods

4.4.1 Induced pluripotent stem cells

Induced pluripotent stem cells derived from adult skin fibroblasts were cultured in the laboratory of Professor Lesley Forrester by Dr Richard Axton, Helen Taylor and others at the Centre of Regenerative Medicine, University of Edinburgh. The development of these cells by Professor Forrester and her laboratory is described in Yang *et al.* (Yang et al., 2017). Essentially, adult skin fibroblasts from donors were transduced with the transcription factors *OCT4, KLF2, SOX2* and *cMYC* (first described by (Takahashi and Yamanaka, 2006)) transforming the cells into pluripotent stem cells capable of differentiation into multiple cell types. The cells were then subject to specific culture conditions to promote differentiation into haematopoietic progenitors
(Jackson et al., 2016, Olivier et al., 2016). Of note, I had no role in the generation or culturing of these cells. Aliquots containing $1x10^6$ iPSC cells at D17 and 23/24 of culture were a kind gift from Professor Forrester and her laboratory and were transported from the Centre of Regenerative Medicine to the King's buildings laboratories for pilot rosetting experiments. Cells were smeared and Giemsa stained, or mixed with MACS purified 9605 and ITvar60 parasites to assess rosetting capability and phenotype (see section 2.1.4, section 2.1.8 and section 2.6.3 for detailed methods, of note, neither parasites nor iPSC were stained with CellTrace™ for these experiments).

The remaining methods refer to cRBC and detailed culture methods are including in section 2.2.

4.4.2 Morphology by cytospin and May Grunwald/Giemsa staining

Samples for cytopsin were prepared by first counting the cells as described in section 2.2.3 then taking sufficient volume to give $1x10^5$ cells per sample and centrifuging at 300g for 4 minutes. The culture supernatant was removed and the pellet washed twice in 100µl of 3% BSA/PBS then resuspended in 100µl of 10% BSA/PBS. Cytospin slides were prepared using a Thermo Scientific™ Cytospin™ 4 Cytocentrifuge (A78300003). A labelled slide was placed in the slide clip with a filter card on top, followed by the reusable cytospin funnel. Samples were loaded in to the cytospin funnels and centrifuged for 5 minutes at 120g. The slides were removed and allowed to dry for 5-10 minutes before fixing in Methanol for 5 minutes. Slides were stained with 10% Giemsa for 25 minutes, in the same manner as parasite smears, then rinsed and allow to dry before visualisation using brightfield microscopy. Alternatively, slides were first stained for 3 minutes with 10% May-Grünwald stain (VWR chemicals, 10047018, made up with dH_2O), rinsed in water for one minute, then stained with 10% Giemsa (filtered and made up with Giemsa buffer) for 15 minutes. The two stage May-Grünwald-Giemsa approach was demonstrated to me while visiting the Duraisingh laboratory and resulted in slides with better resolution for intracellular structures.

4.4.3 Flow cytometry and IFA

The receptor profiles of mature erythrocytes (from Scottish adult peripheral blood donors as before) and cRBC at various stages of culture were analysed using flow cytometry. CD34, CD71 and GYPA were chosen as markers of maturation through the erythroid lineage. CD34 positivity is

expected to be lost around the proerythroblast stage, CD71 expression increases then falls as the cells develop into reticulocytes, while GYPA expression continues to increase as the cells mature (Table 4-2 and (Wood, 2004). The other receptors, GYPC, CR1, HS, CS, Blood group A antigen, H antigen and CD36 are of interest as potential rosetting receptors. At this early point of my PhD, Band 3 was not considered to be of particular interest therefore was not included. The presence of HS was investigated using both IgM antibody to whole HS, and the enzyme-treatment approached followed by 3G10 antibody labelling discussed in Chapter 3 and detailed below.

Table 4-2 Changes in receptor expression during maturation from CD34+ HSC to mature erythrocytes. Adapted from (Wood, 2004).

	BLAST	PRO	BASO	POLY	RETIC	MATURE
CD34	++	-/+	-	-	-	-
CD71	-	+++	+++	+++	+/++	-
CD36	-	++	+++	++	-/+	-
GYPA/ CD235a	-	+/++	+++	+++	+++	+++
Haemo-globin	-	-	-/+	+	++	+++

PRO = proerythroblast, BASO = basophilic erythroblast, POLY = polychromatic erythroblast, RETIC = reticulocyte.

4.4.3.1 Enzymatic pre-treatment with Heparinase III

Three aliquots of culture containing 1-3x10^5 cells each were removed and treated with Heparinase III (two samples) or mock treatment (the same volumes of PBS, one sample) for 3.5 hours as described in Chapter 3. Samples were washed, then one of the Heparinase III treated samples and the mock treated cells, were incubated with 3G10 antibody. The remaining enzyme-treated cells were incubated with an isotype control (further details below in section 4.4.3.2).

4.4.3.2 Preparation of samples

Sufficient cells to give 1-3x10^5 cells per antibody was washed with PBS then resuspended in 1ml PBS with 1µl of LIVE/DEAD^{TM} Near IR fluorescent reactive dye (Invitrogen L34975) and incubated at room temperature on a rotating wheel for 30 minutes. The cells were then washed twice with PBS/1% IgG free BSA (Sigma, A0336) and resuspended at approximately 2-6x10^6 cells/ml. Fifty µl aliquots containing 1-3x10^5 cells per tube were placed in Eppendorf tubes along with the primary antibody to the receptor under investigation (see Table 4-3) and incubated on ice for one hour. Cells were resuspended by gently flicking the tubes every ten minutes.

Table 4-3 Details of primary and secondary antibodies used. Note: All are mouse monoclonal antibodies.

Primary antibody specificity	Final concentration	Details	Secondary antibody
Unstained control	NA	-	Nil
No primary control (IgM secondary)	NA	-	Goat anti-mouse IgM Alexa 488 conjugated (Life technologies, A21042) 1:1000
IgM isotype control	10µg/ml	IgM, Thermo Scientific, 02-6800	
No primary control (IgG secondary)	NA	-	Goat anti-mouse IgG (H+L) secondary antibody, Alexa 488 conjugated (Life technologies, A11029) 1:500
IgG1 isotype control	14µg/ml	IgG1, Abcam, 18443	
IgG2b isotype control	40µg/ml	IgG2bκ, clone MPC-11, Abcam, ab18457	
CD34	14µg/ml	IgG1, BIRMA K3, IBGRL, 9437PA	
GYPA	14µg/ml	IgG1, BRIC 256, IBGRL 9415PAg	
GYPC	14µg/ml	IgG1, BRIC 4, IBGRL, 9417	
CR1	14µg/ml	IgG1, J3D3, Genetex, 44217	
3G10 (HS stub)	40µg/ml	IgG2bκ, clone F69-3G10, Amsbio, 370260	
CD36	20µg/ml	IgG1, FA6-152, Beckman-Coulter, IM0766U	
HS (whole molecule)	10µg/ml	IgM, Clone F58-10E4, Amsbio, 370255-S	Goat anti-mouse IgM Alexa 488 conjugated (Life technologies, A21042) 1:1000
CS (whole molecule)	16µg/ml	IgM, Clone CS-56, Sigma, C8035	
IgG/FITC isotype control	20µg/ml	IgG1/FITC conjugate, Dako, X0927	
H antigen	5µg/ml	IgG1, conjugated to FITC, BRIC 198, IBGRL 9420	
A antigen	20µg/ml	IgG1, conjugated to FITC, BRIC 145, IBGRL, 9434	
IgG/APC control	1:200	IgG1, ebioscience, 17471481	
CD71	1:200	IgG1, conjugated to APC, Clone OKT9, ebioscience, 17071942	

IBGRL = International Blood Group Reference Laboratory, Bristol, UK.

After one hour the cells were washed once with 750µl of cold PBS then resuspended in 50µl of secondary antibody, made up in PBS/1% IgG free BSA (see Table 4-3) containing 1µg/ml of DAPI or Hoechst 0.5mg/ml (Life technologies, H3570). The secondary incubation lasted for 45 minutes on ice, after which the samples were again washed in 750µl of cold PBS. For antibodies which were already conjugated to a fluorophore, the secondary incubation was with 1µg/ml DAPI or Hoechst in PBS/1% IgG free BSA only, with no secondary antibody.

After the final wash, the cells were fixed by resuspending the pellet in 500µl of 0.5% paraformaldehyde (made up in PBS with 16% Formaldehyde solution [w/v] Methanol free, Thermo Scientific, 28908) and incubating for 15 minutes on ice. The tubes were gently centrifuged, the paraformaldehyde removed and the pellet resuspended in 500µl of FACS buffer (PBS/0.5% IgG free BSA/0.02% sodium azide) before being transferred to labelled FACS tubes. Samples were then analysed on a Becton-Dickinson LSR II flow cytometer (BD Biosciences). Depending on the receptor and time point, between three and five different cultures/donors were analysed (including cells derived from adult bone marrow and one umbilical cord). No major difference in results were seen at equivalent times points between donors, with the exception of blood group antigens, in keeping with the different blood groups of the donors.

Once the data had been acquired on the flow cytometer, any remaining sample was centrifuged on to slides using the Cytospin™ 4 Cytocentrifuge as described above, dried and covered with a coverslip mounted using Fluoromount. These slides were visualised using a Leica DM LB2 fluorescent microscope and photographs taken as described previously.

Note: some early experiments with cRBC cells did not use the LIVE/DEAD™ stain.

4.4.4 Recombinant PfEMP1 domain binding to mature erythrocytes and cRBCs

The binding of recombinant PfEMP1 domains (Ghumra et al., 2011, Ghumra et al., 2012) to mature peripheral erythrocytes and filtered, D18 cRBCs was assessed using flow cytometry and IFA.

One million cells per PfEMP1 domain/ were used and day 18 cRBCs were filtered prior to the binding assay using the method described in section 2.2.4. Cells were washed with PBS/1% IgG free BSA, resuspended in 22.5µl of the same and 2.5µl of recombinant PfEMP1 domain added giving the final concentrations listed in Table 4-4.

Table 4-4 Recombinant PfEMP1 domains, primary and secondary antibodies

Recombinant protein	Approx. final concentration	Primary antibody (rabbit number)	Secondary antibody
9197var5 NTS-DBLa (negative control)	480µg/ml	Anti- 9197v5 NTSDBLα (25022)	Goat anti-rabbit IgG Alexa Fluor-488 1:1000 (Invitrogen, A11034)
ITvar09 didomain	170µg/ml	Anti-NTS DBL1α (ITvar09) (5776)	
ITvar09 DBL2γ	350µg/ml	Anti- DBL2γ (ITvar09) (4772)	
ITvar60 NTS-DBL1α	80µg/ml	Anti-NTS DBL1α (ITvar60) (6217)	
TM284 NTS DBLα	216µg/ml	Anti NTS DBL-1 TM284 (6219)	
11019 NTS DLBα	200µg/ml	Anti 11019 DBLα (26886)	
9197var15 NTS-DBLα	125µg/ml	Anti 9197v15 (26888)	
None (No protein control)	NA	Anti-NTS DBL1α (ITvar60) (6217)	

The cells and proteins were incubated on ice for one hour then washed twice with cold PBS before being resuspended in 22.5µl of PBS/0.1% BSA and 2.5µl of the corresponding primary antibody (Table 4-4). The cells were then incubated for 45 minutes, washed twice with PBS and incubated for a further

45 minutes with 50µl of the 1:1000 secondary antibody. The cells were again washed twice with cold PBS then fixed and prepared for flow cytometry as detailed in 4.4.3.2. Between 25,000 and 100,000 events per samples were counted and the remaining cells were used to prepare Cytospin™ slides for IFA.

4.4.5 Rosetting assays with cRBC

Rosetting assays were carried out as described in 2.6.4. Four parasite lines were chosen, namely ITvar60, R29, 11019 and 9605, all cultured in blood group O. Rosetting to TM284 was also assessed for two donors at different time points. ITvar60 and R29 were chosen as they are well-characterised parasites lines with R29 being of particular interest due to its dependence on CR1 for rosetting, while ITvar60 relies less on CR1 (Rowe et al., 1997, Rowe et al., 2000). The other two lines, 11019 and 9605 probably provide a better representation of parasites causing severe malaria in Africa and were used for this reason.

It is important to note that the 'control' blood (normal peripheral blood) was not matched to the cRBC donor and in some cases was a different blood group. Ideally, matched mature peripheral erythrocytes would have been purchased for each bone marrow donor, however this was not possible for practical reasons. All control blood was from blood group O Scottish blood donors and had been prepared and stored as detailed in section 2.1.3.

Matured cRBCs (D18-25 of culture) from three separate adult bone marrow donors (table 2.2: donor numbers D001004150, B+; D001003739, O+ and D001004313, unknown) and one umbilical cord donor were tested for their ability to form rosettes. In addition, cRBC from donor D001004150 were also tested at day 7/8, 11, and 13/14 or 15, with each time point roughly corresponding to the different stages of culture (see Table 2-3).

4.4.6 Generation of Fabs and rosette disruption assays

In order to further investigate the role of GYPA, GYPC and Band 3 in rosetting, antigen-binding fragments (Fab) of the relevant antibodies were generated and tested in rosette disruption assays. It was necessary to use Fab fragments rather than the whole antibody as pilot experiments showed that whole antibody caused massive agglutination of erythrocytes. Of the Band 3 antibodies available (see Table 5-3 for further details), BRIC 6 and BRIC 14 were chosen for the rosette disruption experiments. This is because BRIC 6 antibodies fail to react with erythrocytes with the South Asian Ovalocytosis mutation, which is known to protect against severe malaria and therefore seemed an interesting candidate to study further. The BRIC 14 antibody is directed against the Wright^{b} antigen and pilot experiments by an undergraduate Honours student, Eden Taylor, whom I helped supervise, had shown it appeared to cause rosette disruption in ITvar60, while the other Band 3 antibodies had no effect.

4.4.6.1 Generation of Fab, buffer exchange and concentration of Fabs

The Fab fragments were generated using a Pierce^{TM} Fab Micro Preparation kit from Thermo Scientific (44685), which uses immobilized Papain to digest the whole IgG antibody into Fab and Fc, then purifies the Fabs by binding the undigested antibody and Fc to immobilized Protein A (Figure 4-4). The manufacturers guidelines were followed with the modification that Protein A binding buffer (Thermo scientific, 1861620) was used to wash the digested sample and equilibrate the Protein A immobilization columns. This was recommended by the manufacturer to improve the purification process for mouse IgG1 samples.

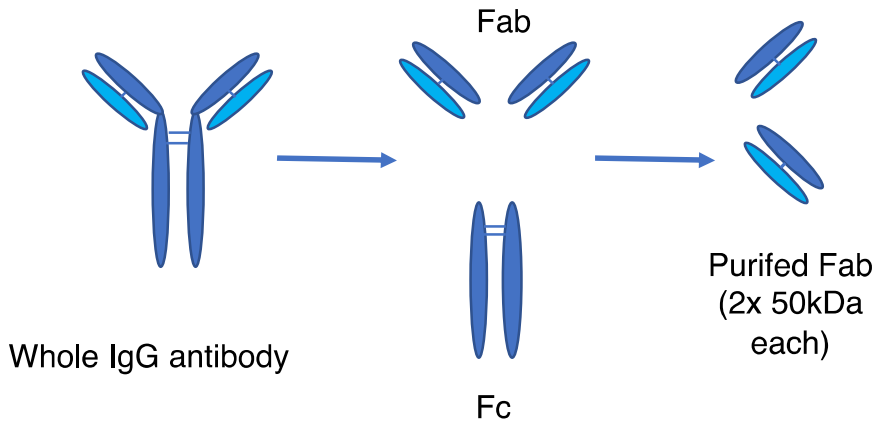


Figure 4-4 Generation of Fabs. The whole IgG antibody is first digested by Papain into Fab and Fc portions. The digested antibody is then passed through a protein A binding column which binds undigested antibody and the Fc portion, allowing the Fab to pass through.

As the resulting Fab were contained in Protein A binding buffer, it was necessary to buffer exchange the sample into PBS before further use. This was done using Zeba^{TM} spin desalting columns (Thermo scientific, 44685) which were first equilibrated with PBS by adding 300µl of PBS to the column, then centrifuging at 1500g for one minute, and repeating this process 3 times. The sample containing Fab in Protein A binding buffer was then added to the top of the column and centrifuged for 2 minutes at 1500g.

The resulting Fab solution in PBS was then concentrated using an Amicon® Ultra 0.5 centrifugal filter device. The device was first equilibrated with 150µl distilled water by centrifuging at 14,000g, then up to 500µl of sample was added and centrifuged for 8 minutes at 14,000g. The device was then turned upside down in a fresh centrifuge tube and centrifuged for 2 minutes at 1200g to collect the concentrated Fabs. The concentration of Fab was measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific).

4.4.6.2 Confirmation of Fab generation by SDS PAGE

Reduced Fab samples were run on SDS PAGE to confirm that fragments were of the expected size (around 25kDa for reduced samples). Approximately 0.4µg (2µl) of Fab was added to 2.5µl of NuPAGE® LDS

sample buffer (4x, Invitrogen, NP0008), 2.5µl DTT (0.1M, Invitrogen, Y00122) and 3µl dH_20, and heated at 70°C for ten minutes to reduce the sample. A NuPAGE® Bis Tris Mini gel (4-12%, 1mm, 12-well, Novex, IM-8042) was rinsed with dH_2O then placed in an electrophoresis chamber (Novex Mini cell xCellsure lock™, Invitrogen, EI0001) filled with MES running buffer (Novex, NP0002). Five µl of Precision Plus Protein™ All Blue Prestained Protein Standard (Biorad, 161-0373) was pipetted into the first well, followed by a reduced sample of the whole IgG antibody as a reference, then the Fab samples. Any empty wells were filled with 3-4µl of samples buffer and 500µl of NuPAGE® antioxidant (Invitrogen, NP0005) was added to the inner chamber. The gel was run at 200V for approximately 35 minutes after which it was removed from the plastic casing and rinsed three times by leaving in a container of fresh dH_2O for 5 minutes each time on a table top rocker. The final wash was discarded and the gel covered with InstantBlue Coomassie stain (Expedeon, ISB1L) and left on the rocker for one hour before a final wash with dH_2O. Images of the gel were taken using an iPhone 5 camera.

4.4.6.3 Rosette disruption with Fabs

Rosette disruption assays were carried out as described in section 2.6.2. The test samples are listed below:

1. **GYPC** BRIC 4 Fab (IBGRL, 9417) final concentration 100µg/ml
2. **GYPA** BRIC 256 Fab (IBGRL 9415PA) final concentration 100µg/ml
3. **Band 3** BRIC 6 Fab (IBGRL 2618L) final concentration 100µg/ml
4. **Anti Wright^b** BRIC 14 Fab (IBGRL, 9413PA) final concentration 100µg/ml
5. **Positive control:** Fucoidan (Molekula, 9072-19-9) final concentration 100µg/ml OR 1mg/ml heparin (Sigma, H4784, diluted with PBS) for 11019 and 9605.
6. ***Treatment free*** tube, PBS only- as negative control

7. ***Isotype control:*** Fab of IgG mouse isotype control, (Abcam, 18443) final concentration 100µg/ml

Due to limited volume of concentrated Fab, the final total volume of parasite culture and Fab was 10µl (approximately 3µl of Fab with 7µl of parasite culture). Three independent experiments were carried out for each parasite strain and every experiment was blinded. Rosette frequency was calculated from the mean of two separate slides on which 200 infected cells per slide were counted. Statistical analyses were carried out using GraphPad Prism software (Version 7.0, La Jolla California, USA, www. Graphpad.com). Comparisons were made using an Ordinary one-way ANOVA with Dunnett's multiple comparisons test with p value of <0.05 considered to be significant.

4.4.7 Lentiviral knockdown of cRBC

Attempts were made to knockdown GYPA, GYPC and CR1 in cRBC using transduction via spinoculation with short hairpin RNA lentiviral particles, in a similar fashion to that described in (Lee et al., 2014, Niang et al., 2014). Ready-made lentiviral particles, all of which permit the use of Puromycin for selection, were purchased from Santa Cruz biotechnology as listed below:

1. Control shRNA Lentiviral Particles-A (Santa Cruz Biotechnology, sc-108080). A scrambled shRNA negative control, which should not affect receptor expression but confers Puromycin resistance.
2. cop GFP Control Lentiviral Particles (Santa Cruz Biotechnology, sc-108084). A control which should not affect receptor expression but introduces the green fluorescent protein gene, causing the cells to fluoresce, which was used to check the spinoculation method.
3. Glycophorin C shRNA (h) Lentiviral Particles (Santa Cruz Biotechnology, sc-42884-V)
4. CD35 (CR1) shRNA (h) Lentiviral Particles (Santa Cruz Biotechnology, sc-29994-V)

5. Glycophorin A shRNA (h) Lentiviral Particles (Santa Cruz Biotechnology, sc-42882-V)

According to the datasheets provided by Santa Cruz Biotechnology, the shRNA lentiviral particles contain a pool of transduction-ready lentiviral particles and 3 constructs targeted to the gene of interest which contain 19-25 nucleotides, though exact details of the target sequences are not provided.

cRBC were cultured until day 6 or 7 then pelleted and resuspended in incomplete RPMI at a concentration of $1x10^6$ cells/ml. In parallel, the lentiviral particles were thawed and a 96-well plate blocked with a 100µl per well of PBS/1%BSA for 20 minutes. The block was removed and 100µl of cell suspension ($1x10^5$ cells) was pipetted into the well. To this was added: 10µl of lentiviral particles, 90µl of incomplete media, and 0.8µl of Polybrene (Santa Cruz Biotechnology, sc-134220, diluted to 1mg/ml with PBS). This resulted in a multiplicity of infection (MOI, the number of transducing lentiviral particles per cell) of 0.5. My earlier experiments comparing an MOI of 0.5, 1 and 5 (appendix 1) had shown no benefit to a higher MOI and discussions with the Duraisingh laboratory who have experience of this method suggested than an MOI of 0.5 was appropriate.

The cell/lentiviral particle mix was then centrifuged for 2 hours at 1000g at room temperature; this ‘spinoculation’ method has been shown to enhance the *in vitro* infectivity of various viruses (O'Doherty et al., 2000, Guo et al., 2011, Yan et al., 2015). The plate was then allowed to rest at 37ºC for one hour before being washed with incomplete then complete media, and placed back into culture at a cell density of $2x10^5$ cells/ml. After 24 hours, 2µg/ml of Puromycin (Calbiochem 540411) was added to the culture medium to select for transduced cells. Puromycin was added at every media change up to and including day 11.

At day 14-17 of culture, cells were checked for receptor or GFP expression using flow cytometry as described in section 4.4.3, except the secondary antibody used was an anti-mouse IgG tagged with Alexa 594 (1:1000, Invitrogen, A11032), to avoid confusion with the GFP expression in controls, and cells were analysed on the BD LSRFortessa^{TM} flow cytometer (BD Biosciences).

4.5 Results I: Induced pluripotent stem cells

Induced pluripotent stem cells derived from skin fibroblasts and cultured for 17-24 days in culture medium and cytokines designed to promote maturation towards the erythroid pathway were stained with Giemsa. Staining revealed a morphologically diverse cell population as shown in Figure 4-5. All cells had a nucleus and were of varying size and granularity.

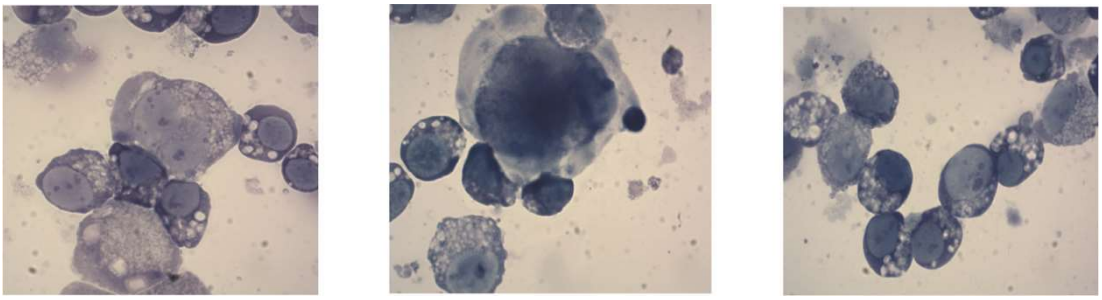


Figure 4-5 iPSC after 17 days of culture stained with Giemsa

When used in rosetting assays the iPSC derived cells did exhibit a form of cytoadherence, which is some cases resembled rosetting (Figure 4-6A). However, some of the larger cells demonstrated a very different phenotype, with the iPSC derived cells becoming coated with multiple infected erythrocytes (Figure 4-6 B and C). While this does not look like true rosetting, the images are very similar to that of an activated white blood cell covered with infected erythrocytes shown in (Wahlgren et al., 1995). Of note, the identify of these larger cells was not investigated further and cells were not cultured further in our laboratory.

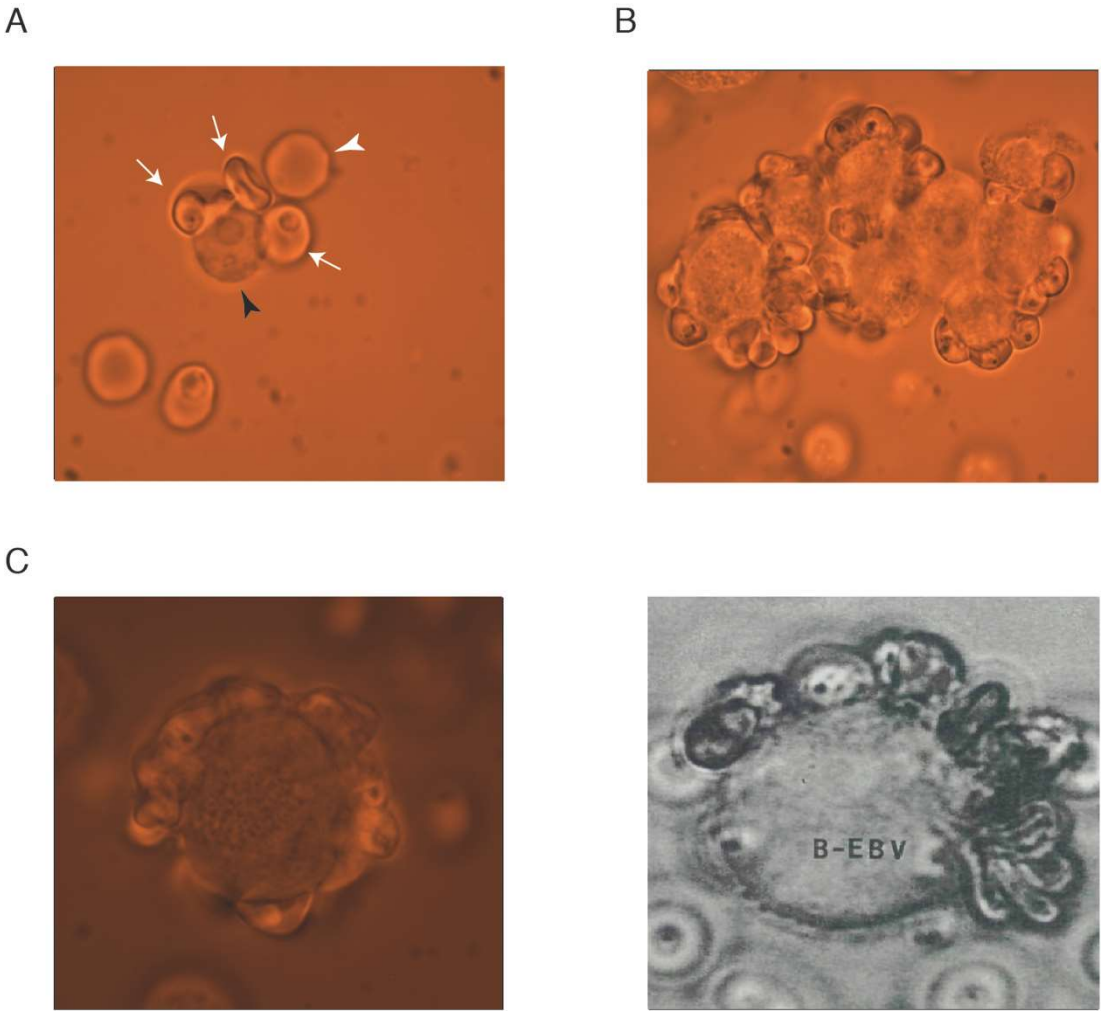


Figure 4-6 iPSC with purified ITvar60 parasites. A shows a rosette-like formation consisting of an iPSC derived cell (black arrow-head), three infected erythrocytes (white arrows) and an uninfected erythrocyte (white arrowhead). B shows a cluster of large iPSC derived cells coated with infected erythrocytes. C a comparison of a similar, large iPSC derived cell (LEFT) to an image of an activated B cell coated with infected erythrocytes published in (Wahlgren et al., 1995)

Taken together, these small, pilot experiments suggest that the population of cells generated from this form of iPSC culture are very heterologous and may not be suitable for the type of experiments investigating erythrocyte receptors which I intended to conduct.

4.6 Results II: cRBC derived from adult bone marrow HSC

4.6.1 Expansion and maturation of HSC into enucleated erythrocytes

CD34+ haematopoietic stem cells derived from adult bone marrow were successfully cultured into immature reticulocyte-like cells with enucleation rates up to 92%. Figure 4-7A shows the typical growth curves for the cells in terms of the fold change in cell numbers between each time point. Figure 4-7B shows the fold change from day zero and absolute cell numbers; note that the data showed in this figure are projected numbers based on the fold increased measured in Figure 4-7A as cells were constantly being used for experiments. Growth was maximal between days 4 and 8, and cell number increased until day 15-18, after which there was a significant drop off due to cell death.

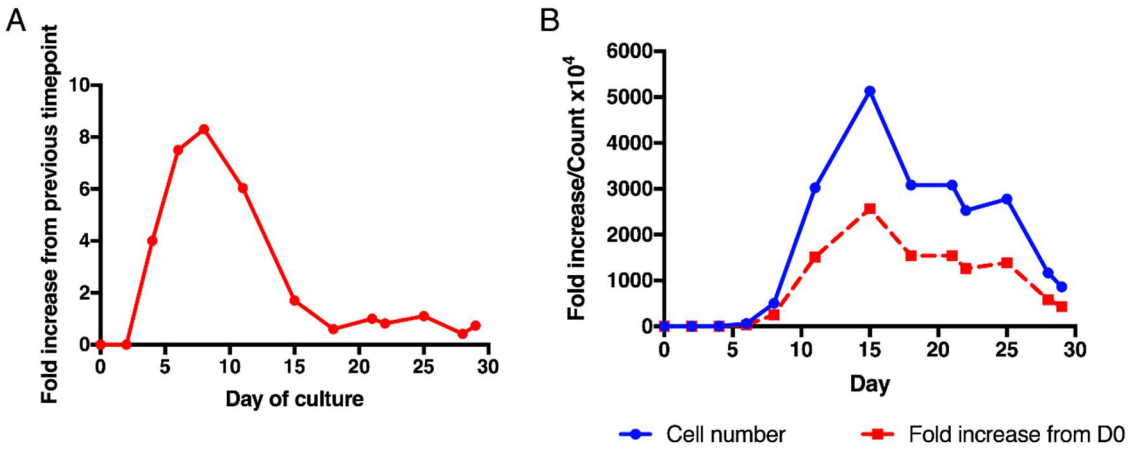


Figure 4-7 cRBC growth. A Fold increase in cRBC cell numbers compared to the previous time point (usually about 48 hours). B Projected total cRBC cell numbers (x10^4) over time if no cells are used for experiments (solid blue line) and fold increase from day 0 of culture (red dashed line). Both graphs show data from donor 1001448

The morphology of the cells matured over the 18 or more days of culture from large, nucleated cells, to haemoglobin producing, mostly enucleated cells (Figure 4-8). The enucleation rate at maturity (day 18) ranged from 76-92% of unfiltered cells. Filtering produced a homogenous population of

enucleated cells (Figure 4-8), however despite the high number of enucleated cells prior to filtering, around 80-90% of the cells were lost during the filtration process, presumably due to lysis.

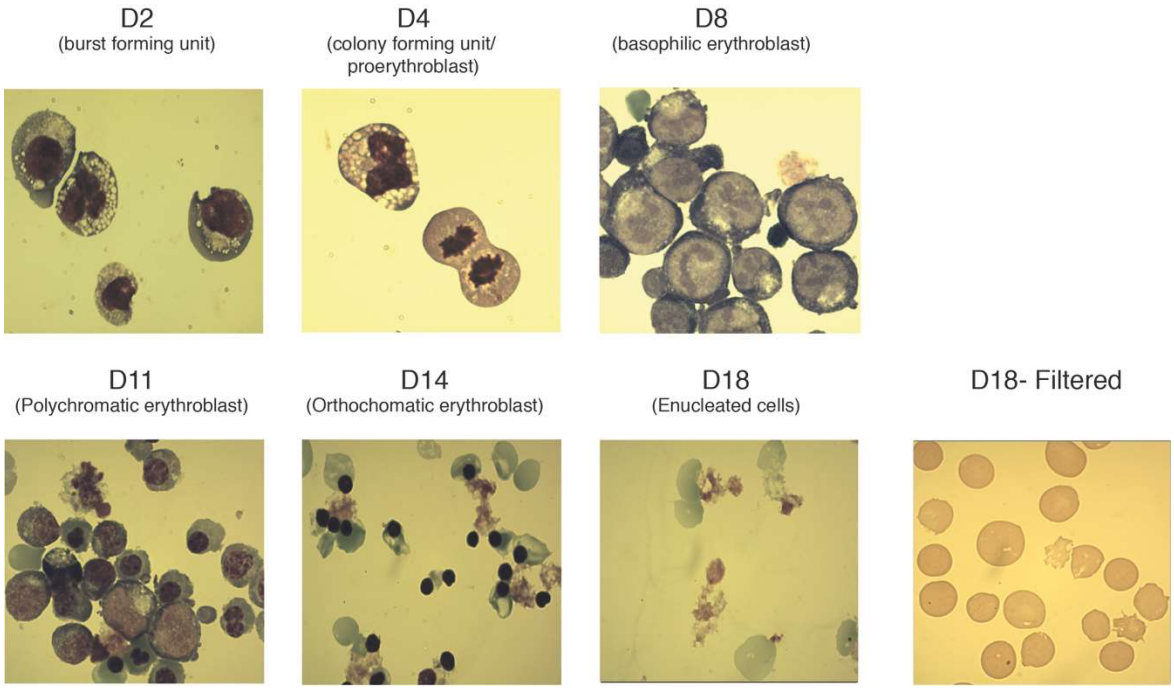


Figure 4-8 Morphology of maturing cRBC over an 18-day culture period. Cells were stained with May Grunwald/Giemsa as described in the methods. The filtered cells from Day 18 were passed through a filter disc to remove nucleated cells as described in section 2.2.4. Note that unfiltered cultured from D14 and D18 contain the extruded pyrenocytes and debris from dead/dying cells.

The cell pellet was noted to turn red, indicating the production of haemoglobin, between day 8 and 11 (Figure 4-9).

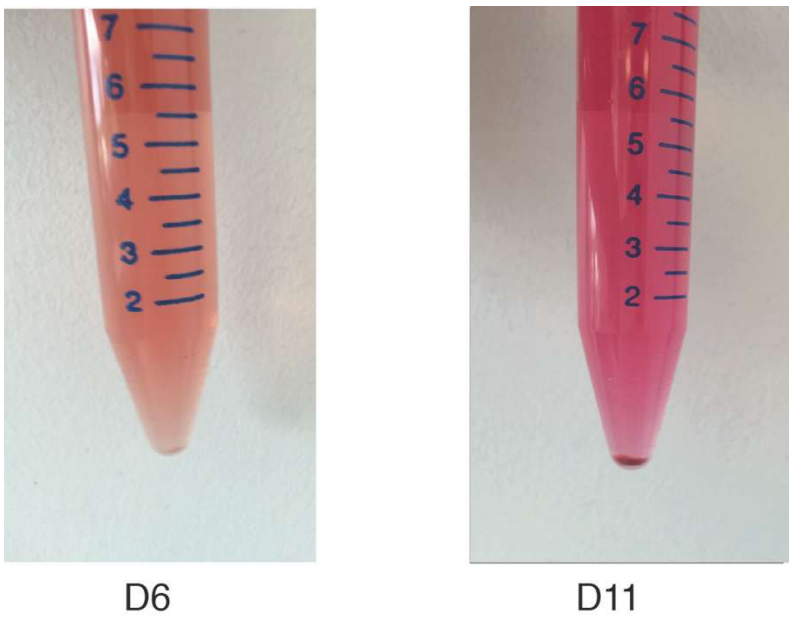


Figure 4-9 Pelleted cRBC at day 6 and 11 of culture. the red colour of the pellet on D11 indicating the production of haemoglobin

The evolving size and nuclear staining profile can be seen in the flow cytometry plots of Figure 4-10, which also shows the gating strategy used for the receptor profile experiments.

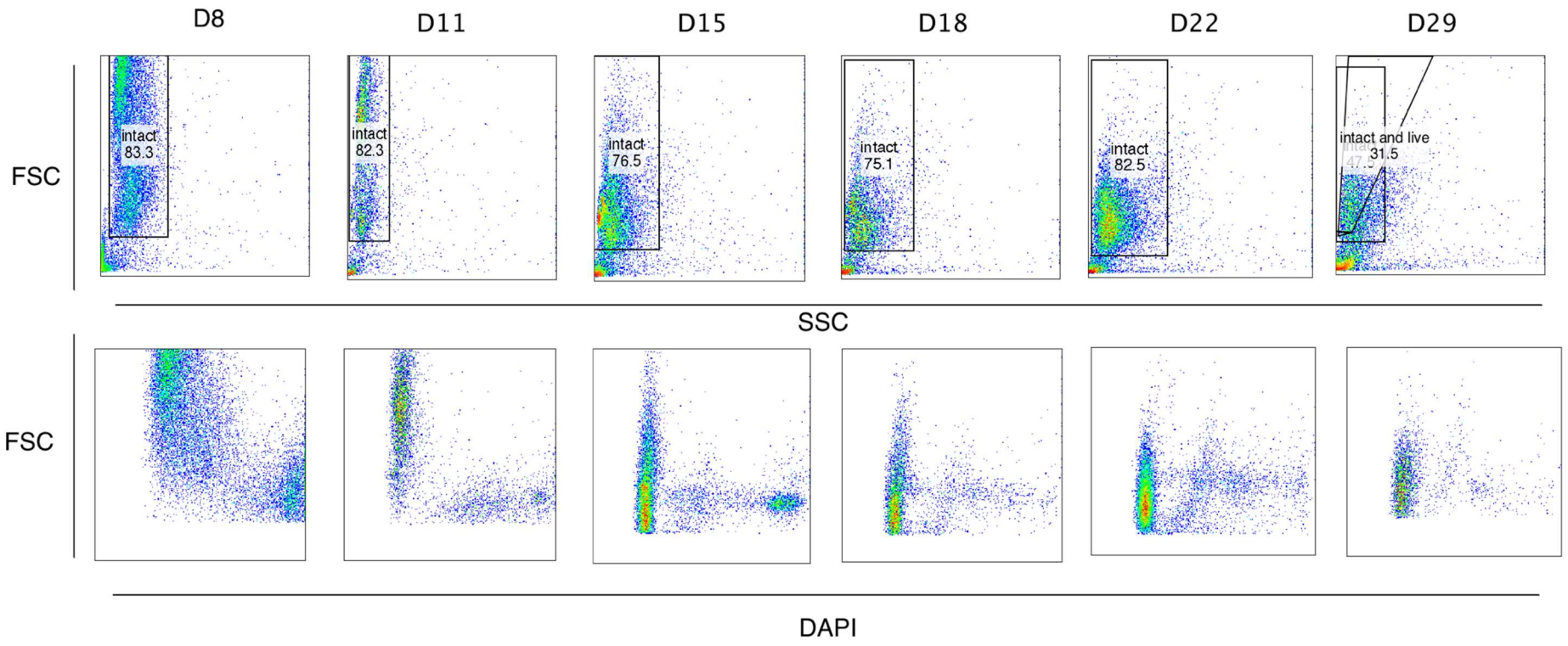
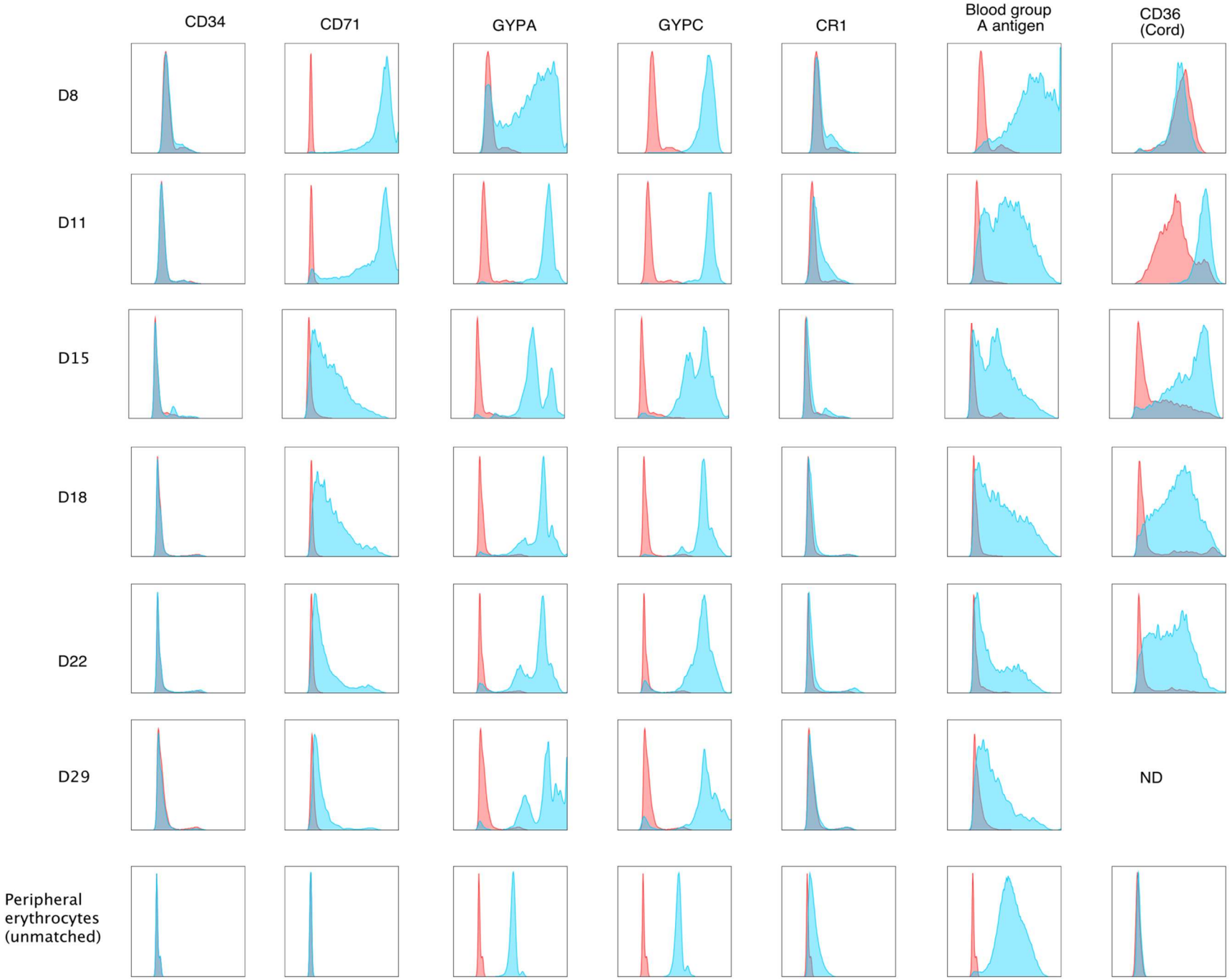
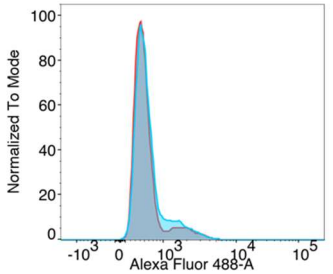


Figure 4-10 Flow cytometry plots evolve with increasing cRBC maturity. The FSC/SSC (UPPER) and nuclear staining (LOWER, stained with DAPI) patterns evolved over time from large, heterogenous, nucleated cells, to smaller, homo, mainly enucleated cells.

4.6.2 Cell surface receptor expression over time

As the cRBCs matured, the cell surface receptor expression changed as expected (Figure 4-11). CD34+ expression was lost very quickly and could only be detected on day 4 (Figure 4-12), while GYPA and CD71 expression increased greatly. CD71 decreased between D18 and D29, but was still expressed to a fairly large degree, indicating that cRBC can, at best, represent relatively immature reticulocytes. CR1 expression remained noticeably low across all the donors tested. CR1 expression can vary significantly between individuals (Moulds et al., 1992), therefore these results could simply due to naturally low levels of CR1 expression from the donors tested. However, lower CR1 level have been previously documented on cRBC compared to peripheral erythrocyte samples (Dankwa et al., 2017). The progressive loss of heparan sulfate with cRBC maturity has been previously described in Figure 3-16.





BLUE: Receptor
RED: Isotype control

Figure 4-11 cRBC receptor profile over time. The larger graph on the right shows the axes. RED= isotype control primary antibody and BLUE = receptor of interest. The bottom panel shows the receptor profile of mature peripheral erythrocytes of blood group A. ND= not done. The gating strategy for these plots is shown in Figure 4-10

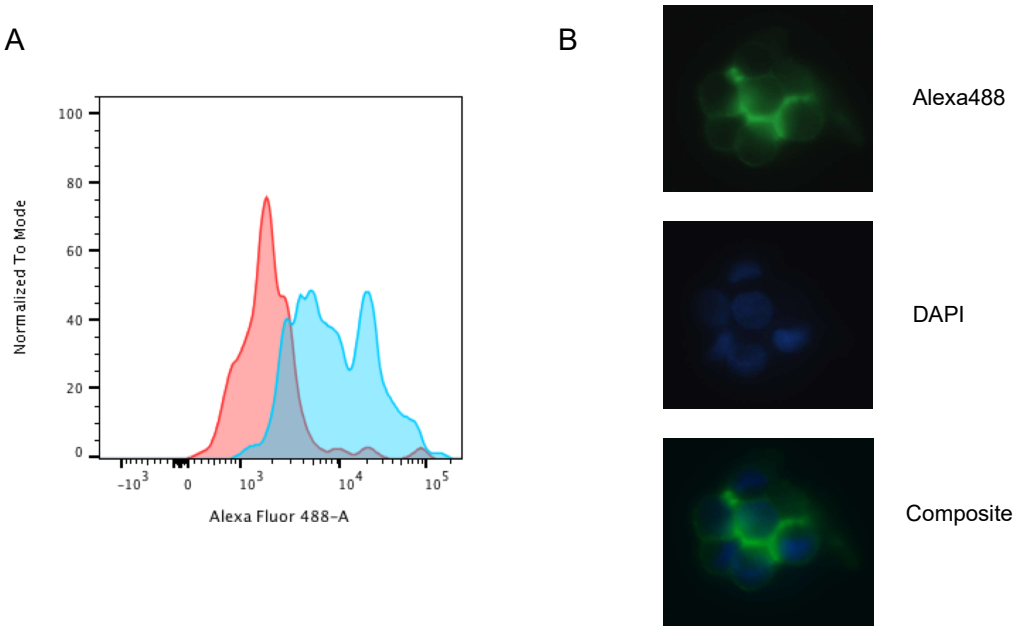


Figure 4-12 CD34 expression on cRBC on day 4 of culture. A flow cytometry plots of D4 cRBC, RED- IgG isotype control, BLUE- CD34+. B IFA of CD34+ CRBC on D4, Green Alexa 488 tagged secondary antibody binding to anti-CD34 primary, and blue DAPI stained nuclei. Composite image generated using Image J without further image manipulation.

4.6.3 Recombinant PfEMP1 domain binding to mature erythrocytes and cRBC

Recombinant PfEMP1 domains from rosetting variants bound equally well to mature donor erythrocytes and filtered cRBC at D18 of culture (Figure 4-13 A and B respectively). Of note, the ITvar09 didomain (derived from parasite strain R29) and ITvar60NTS DBLα (from ITvar60) showed particularly bright fluorescence on the cRBCs, a finding of particular interest when taken in context with the results of the cRBC rosetting assays with these parasites (section 4.6.4).

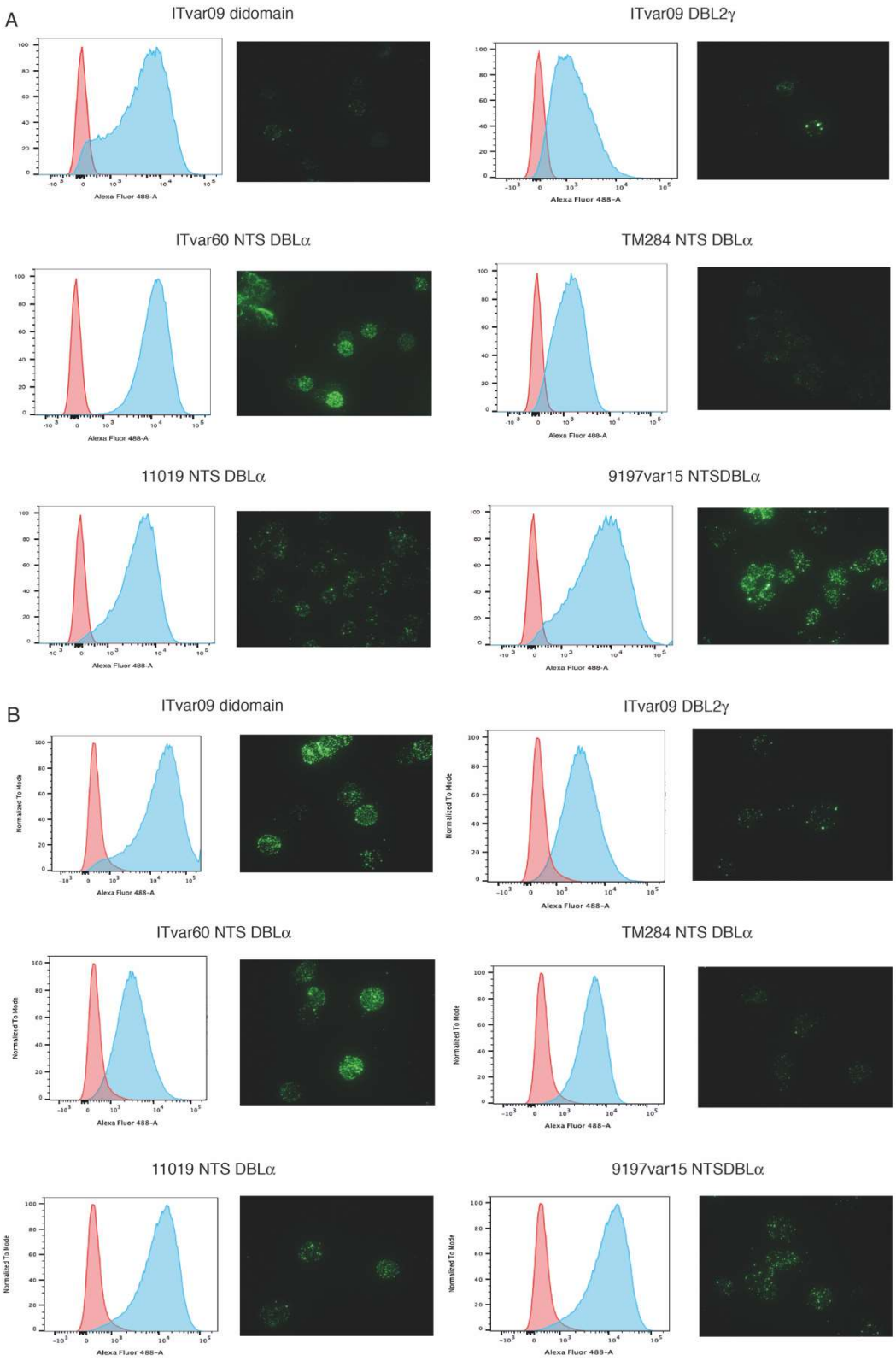


Figure 4-13 Binding of rPfEMP1 to mature erythrocytes (A) and filtered D18 cRBC (B). Flow cytometry plots- RED = 9197var5 NTS-DBLα (negative control), BLUE = rPfEMP1 binding, all normalized to mode. IFA- Green Alexa 488 showing binding of the rPfEMP1 to uninfected mature erythrocytes or D18 cRBC, visualised using the x100 objective.

4.6.4 Variation in ability of cRBC to rosette with *P. falciparum* infected erythrocytes

The rosetting ability of cRBC after 18-25 days of culture from 4 independent donors (including one cord donor) were tested with parasite lines ITvar60, R29, 11019 and 9605, two donors were also tested used TM284. For reference, the cRBC from experiment 3 in Figure 4-14 are the same as those tested for rPfEMP1 binding in Figure 4-13B (Donor no. D001004313). Interestingly, given the excellent binding of the ITvar60 and R29 rPfEMP1 domains, matured cRBCs showed very poor rosetting with purified parasites of ITvar60 and R29 (Figure 4-14). Rosetting frequency with 11019, 9605 and the single donor tested at day 18 with TM284 was close to that seen with unmatched peripheral donor erythrocytes (Figure 4-14, no significant differences based on an unpaired t-test, and Figure 4-16). Of note, the 'control' peripheral donor erythrocytes were not matched to the bone marrow donor from whom the cRBC were derived. All the peripheral erythrocytes were of blood group O, while the cRBCs varied and were not known in all cases as showed in Figure 4-14.

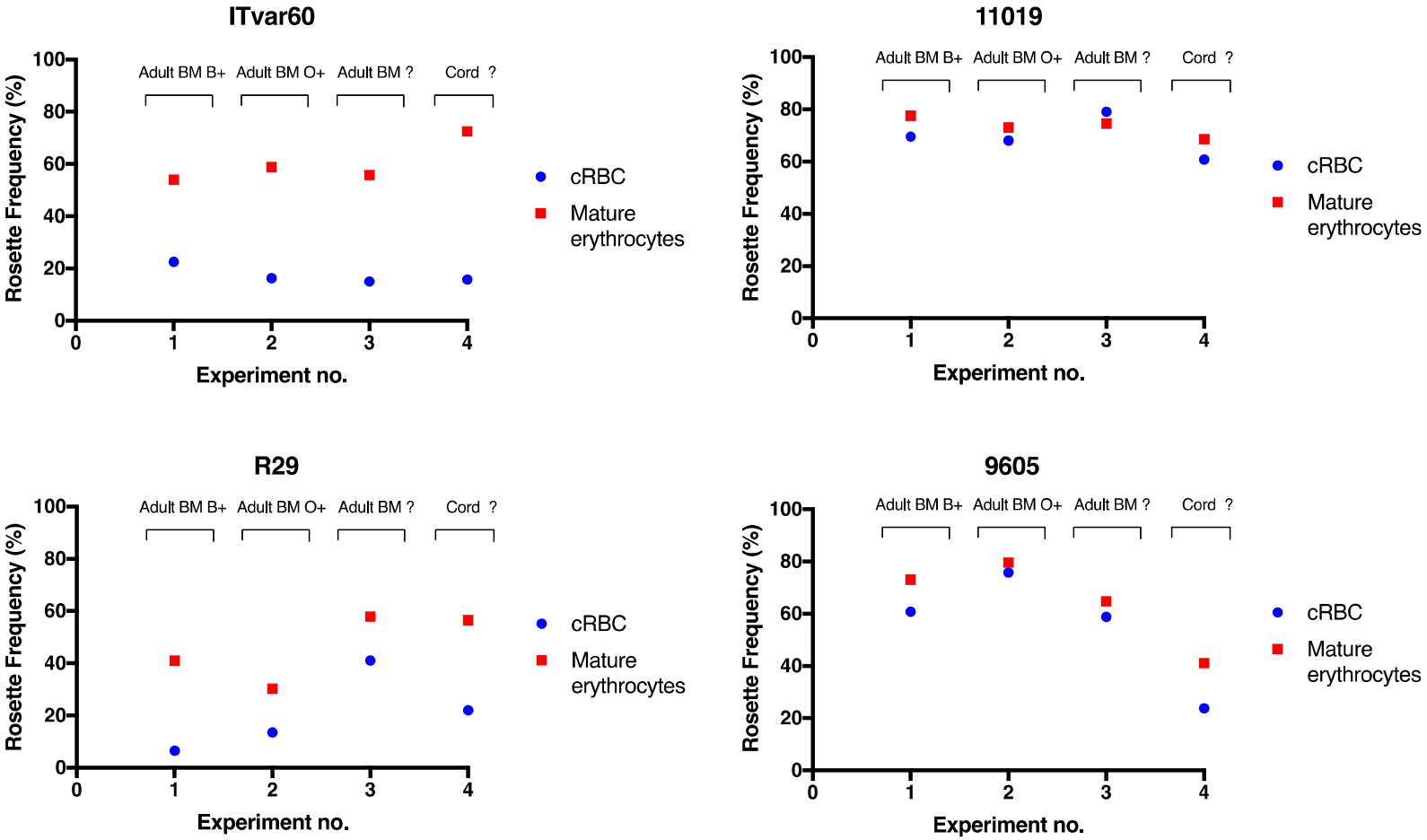


Figure 4-14 Rosetting of unfiltered D18-25 cRBC with various parasite lines. cRBC derived from either adult bone marrow (BM) or a single umbilical cord donor were cultured and tested between day 18 and 25 of culture. The origin and blood group of the cRBC (if known) is indicated above the data points. Of note the mature erythrocytes were unmatched and all of blood group O+. Each data point represents the mean of two technical replicates counted from two different slides. BM= bone marrow, ? = blood group unknown.

For one cRBC donor (D001004150, blood group B+), rosetting was tested at multiple stages of maturity. As shown in Figure 4-15, rosetting was poor at all stages of maturation for ITvar60 and R29. 11019 rosetted well even with early stage (basophilic and polychromatic erythroblast) cRBC, while 9605 did not rosette with earlier stages, but this improved as the cells matured.

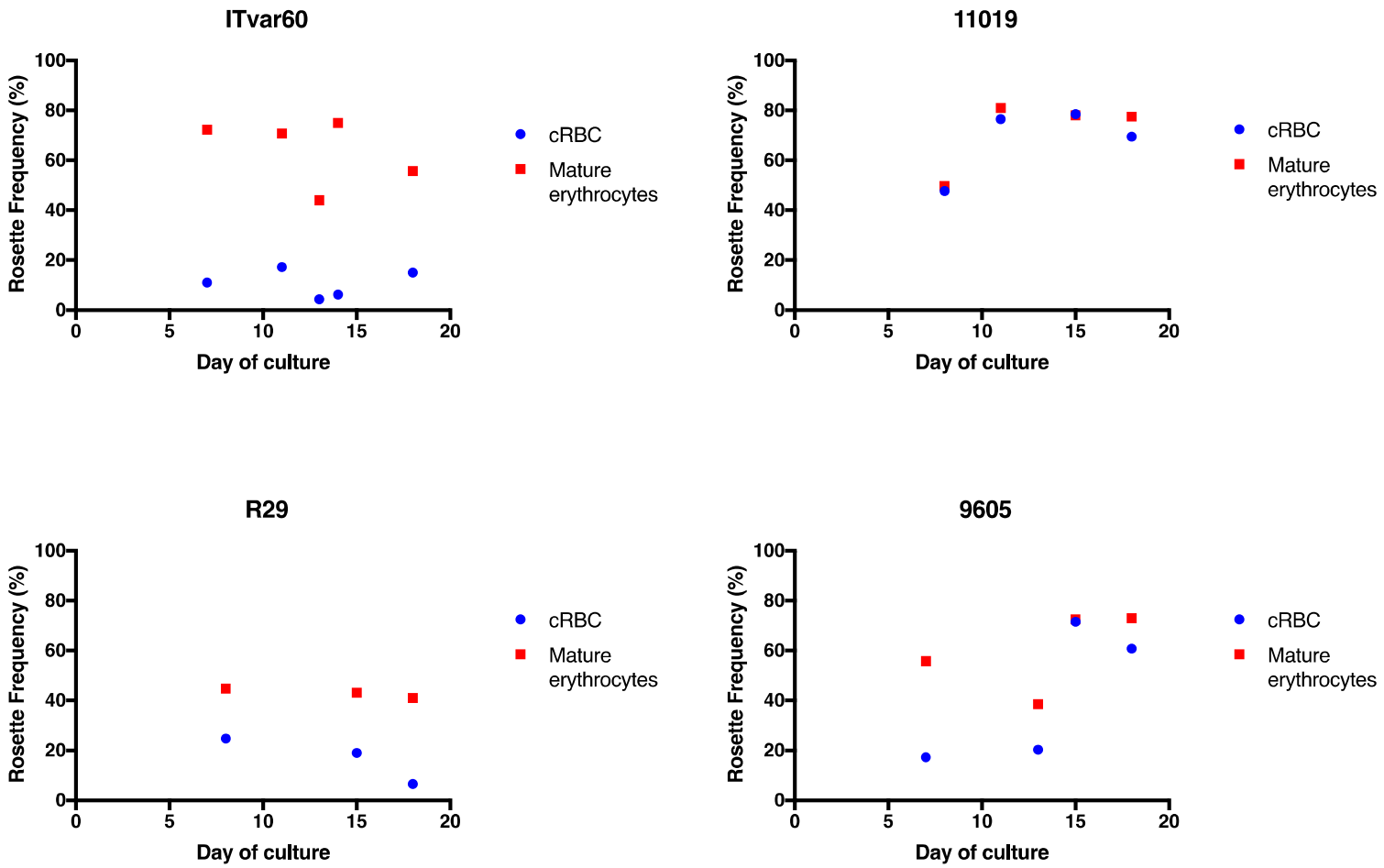


Figure 4-15 cRBC rosetting at different stages of maturity. cRBC from a single adult bone marrow donor (blood group B+) were tested for rosetting a various different time points, broadly representing the different culture stages. Each data point represents the mean of two technical replicates counted from two different slides. Again, the mature erythrocytes were not matched

TM284 was only tested at two time points, day 11 and day 18 using two different donors (Figure 4-16). Further experiments were not carried out with TM284 due to time constraints.

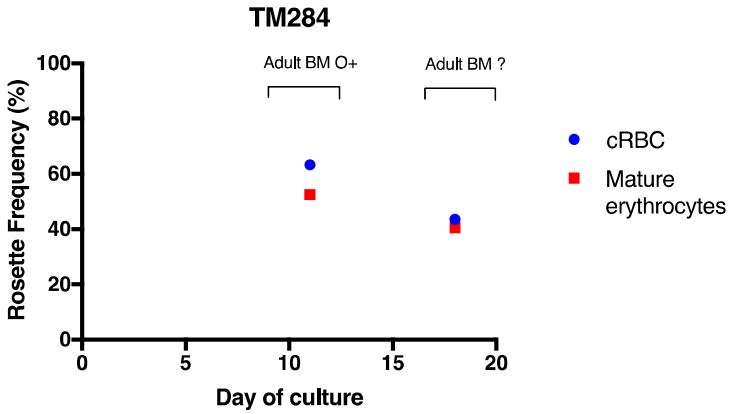


Figure 4-16 Rosetting of cRBC at day 11 and 18 (two separate donors) with TM284. Each data point represents the mean of two technical replicates counted from two different slides.

A further observation made during these experiments was that even for parasites that had a high rosetting frequency with cRBC, there was a qualitative difference in the rosettes themselves. Rosettes with cRBC appeared smaller and looser that those with mature erythrocytes, even though the proportion of rosetting infected cells were equal (Figure 4-17). It is important to note that the cRBC shown in Figure 4-17 are of blood group B, while the mature peripheral erythrocytes are blood group O. However, in general, for parasites which have a preferring blood group for rosetting, rosettes in blood group O are smaller (Barragan et al., 2000b), therefore the discrepancy in size seen between the mature erythrocytes and the cRBC may in fact be even greater than demonstrated here.

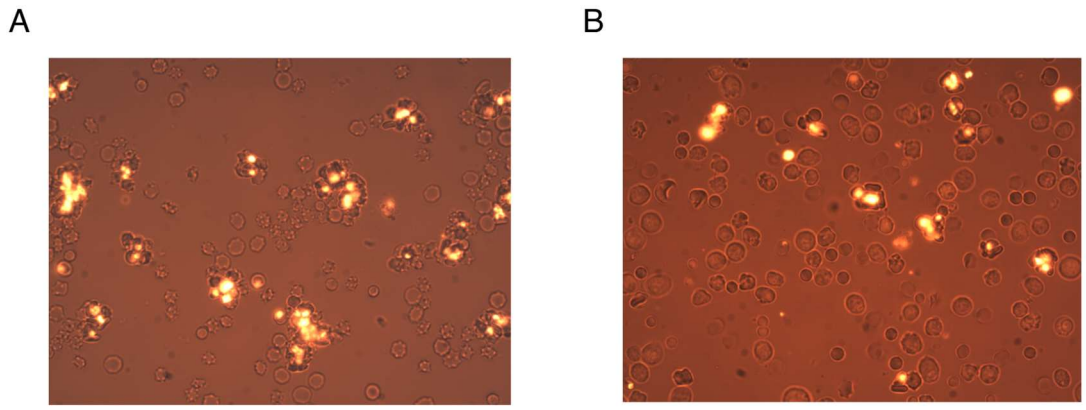


Figure 4-17 Smaller rosettes with cRBC. Parasites from line 11019 were purified and mixed with either A mature peripheral donor erythrocytes (blood group O+) or B cRBC (blood group B+). Infected erythrocytes were stained with ethidium bromide and can be seen as bright yellow/orange. Cells were imaged under combined fluorescence and white light using a x40 objective on a Leica DM LB2 fluorescent microscope and photographed using a Yenway CMOS 5mpx camera. The brightness of the images has been increased equally for both images using Image J software.

4.6.5 Analysis of Dankwa *et al.* comparing cRBC to mature erythrocytes

The variability in rosetting phenotype by strain displayed by the cRBC was unexpected given the excellent rPfEMP1 binding and the similar receptor profile between cRBC and mature erythrocytes. In fact, one might even expect better rosetting given that cRBC express higher levels of some previously proposed rosetting receptors, namely heparan sulfate, in the early stages of cRBC culture, and CD36.

I considered two possible explanations for this; firstly, that the cRBCs are lacking a key receptor(s) that is essential for rosetting for some strains, and has an accessory role in others, or that the cRBC express a receptor that is in some way blocking or interfering with rosetting. To investigate this further, I required more data on the wider receptor profile of cRBC compared to mature peripheral erythrocytes. These data were obtained from a paper published by Dankwa *et al.,* in which the expression of 78 cell surface receptors was quantified on both cRBC and mature peripheral erythrocytes using Tandem Mass Tag mass spectroscopy (Dankwa et al., 2017). In this paper, the receptors were clustered into three groups; those which were

upregulated on cRBC compared to mature erythrocytes, those which were similar and those which were downregulated on cRBC (Figure 4-18).

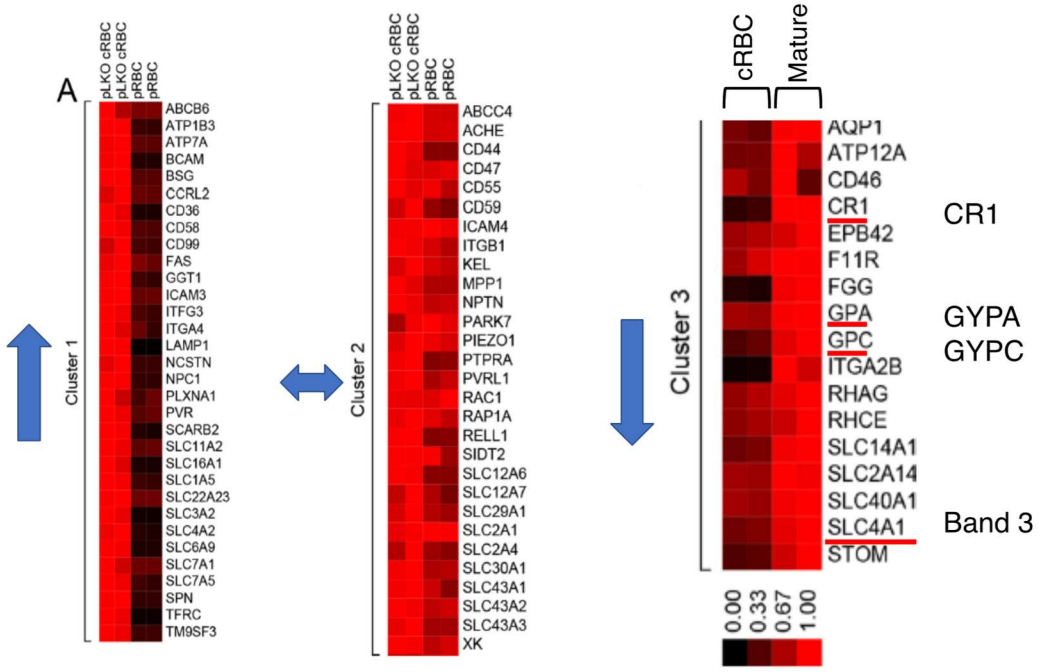


Figure 4-18 Comparison of receptor expression on cRBC and mature erythrocytes. Adapted from (Dankwa et al., 2017). Receptor expression from two, presumably separate, day 17-20 cRBC cultures (referred to in the paper as pLKO cRBC) and two mature erythrocyte donors (pRBC) was quantified using mass spectroscopy. The blue arrows indicate whether receptor expression in cRBC was greater (cluster 1), the same (cluster 2) or less (cluster 3) than peripheral RBC. Receptors of interest have been underlined in red and listed on the far right.

The lack of a certain receptor seemed more plausible than a blocking one, therefore I focused my attention on the receptors which were downregulated in cRBC compared to mature erythrocytes. Of interest in this group were the previously described rosetting receptors CR1, GYPA and GYPC. The lack of CR1 in particular, could explain the poor rosetting for R29 which is highly dependent on CR1 (Rowe et al., 1997), however ITvar60 which is much less reliant this molecule (Rowe et al., 1997, Rowe et al., 2000). Of the other under-expressed molecules, SLC4A1, otherwise known as Band 3, caught my attention as a receptor potentially worthy of further investigation. Autosomal dominant mutations of the Band 3 gene lead to the condition South Asian Ovalocytosis, which is known to protect against severe *P. vivax* malaria (Rosanas-Urgell et al., 2012) and cerebral malaria in *P. falciparum*

(Genton et al., 1995, Allen et al., 1999). I therefore decided to explore the importance of Band 3, GYPA and GYPC for rosetting across a range of parasites strains using rosette disruption with antibody fragments.

4.6.6 Antibodies to Wright^b disrupt rosettes across all tested strains

4.6.6.1 SDS PAGE showing successful generation of Fab

Fab generation was successful and bands of the expected molecular weight of around 25kDa were detected on SDS PAGE as shown in Figure 4-19.

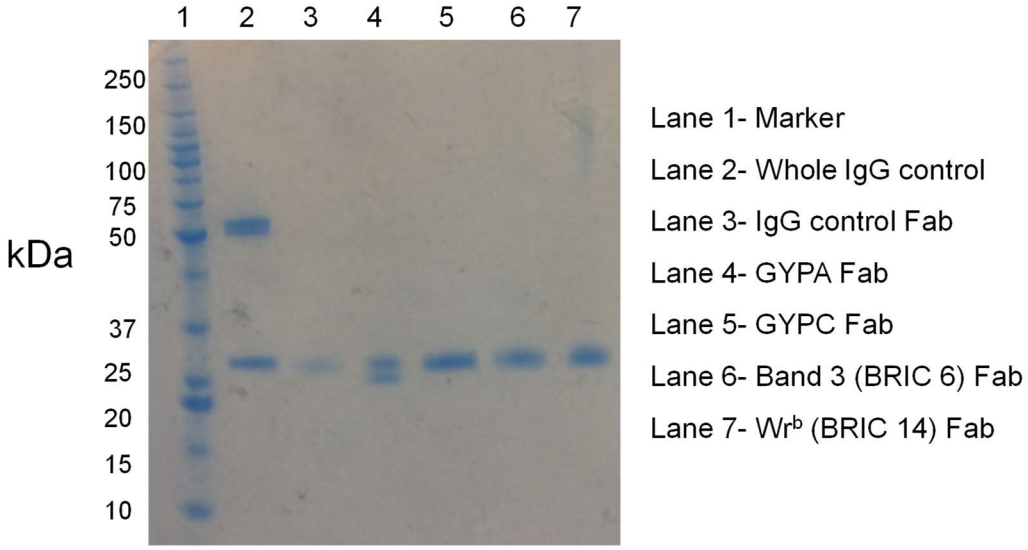


Figure 4-19 Reduced SDS PAGE gel showing Fab at expected molecular weight of around 25kDa. A whole IgG isotype control antibody included in lane 2 shows the reduced heavy chain at around 50kDa and light chain at 25kDa.

4.6.6.2 Wright^b Fabs disrupt rosettes across all parasite strains

The antigen binding fragments of antibodies to GYPA, GYPC, Band 3 (BRIC 6) and the Wright^b antigen (an antigen carried on Band 3 which is closely associated with GYPA, further details below and in Chapter 5) were tested in

rosette disruption assays for six different parasites lines, all cultured in blood group O. The ability of the Fab to disrupt rosettes varied across the different strains, except for the Wright^{b} Fab which significantly reduced the rosetting frequency across all lines tested to levels similar to that of the positive control (Figure 4-20). Neither GYPA, GYPC nor Band 3 (BRIC 6) Fabs showed any effect on rosetting for most of the lines tested, though the range of results was wide for 9605 suggesting that additional repeats may have revealed some effect. For R29 and ITvar60, both GYPA and Band 3 (BRIC6) Fab did show a modest, but statistically significant, level of rosette disruption.

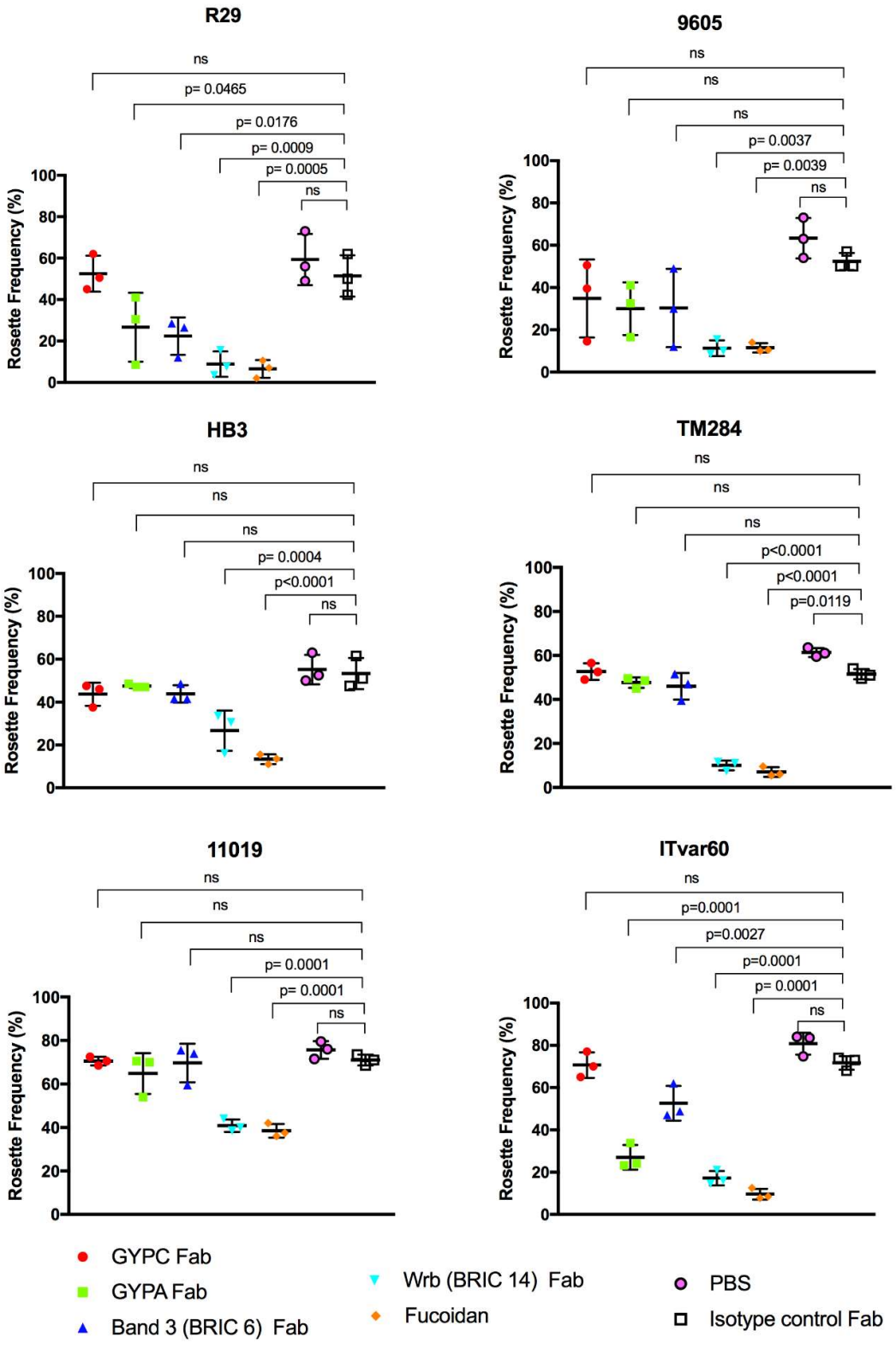


Figure 4-20 Rosette disruption with GYPA, GYPC, Band 3 and Wright^b Fabs across six parasites lines. Each data point represents the results of one independent, blinded experiment (the mean of two technical replicates as described in the text). p-value <0.05 was considered significant. Error bars show the mean and standard deviation of the three results. For strain 11019, heparin 1mg/ml was used as the positive control as rosetting in 11019 is not sensitive to Fucoidan.

Images from the rosettes disruption assays for ITvar60 are shown in Figure 4-21 and demonstrate the marked rosette disruption of the Wright^{b} Fabs, comparable to the Fucoidan positive control, and the more subtle effects of GYPA and Band3 (BRIC 6) Fabs.

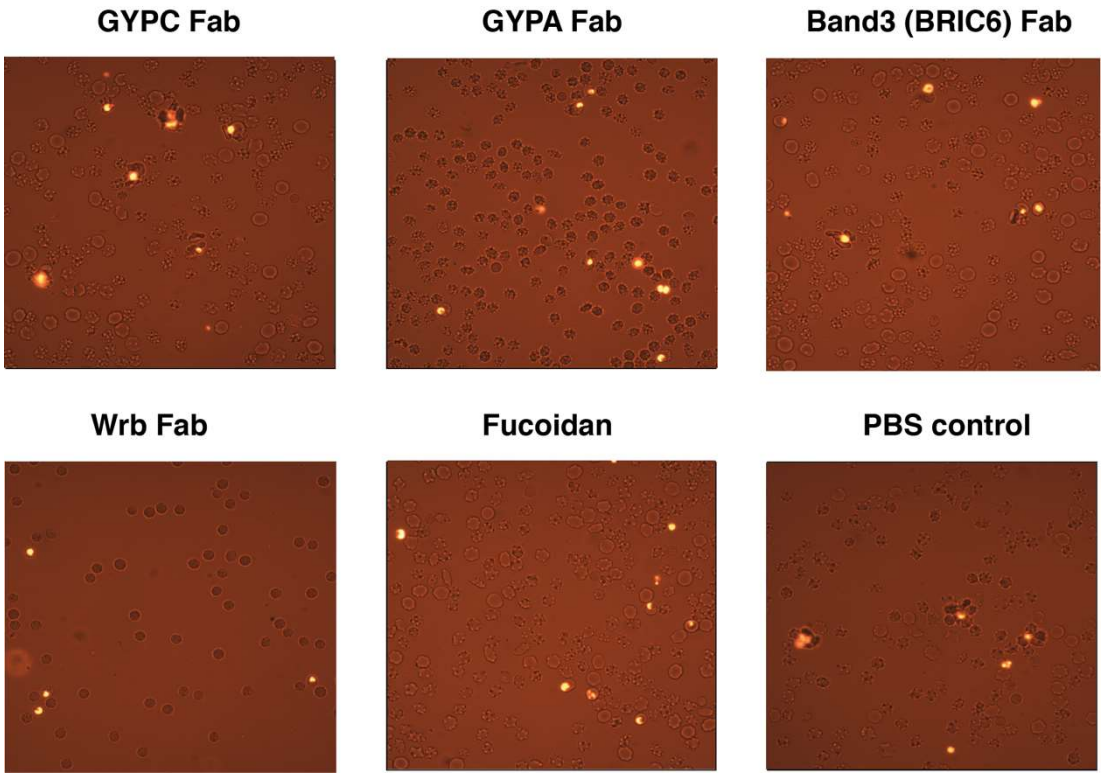


Figure 4-21 Rosette disruption of ITvar60 by Fab fragments. Infected erythrocytes are stained bright orange with ethidium bromide. Cells were imaged under combined florescence and white light using a x40 objective on a Leica DM LB2 florescent microscope and photographed using a Yenway CMOS 5mpx camera. Wrb= Wright^{b}

4.6.7 Lentiviral knockdown cRBC

In order to investigate the role of specific rosetting receptors using an alternative approach, I attempted to generate knockdown cRBC in which the expression of receptors of interest was reduced. This proved technically challenging for a number of reasons, mainly due to the fact that cRBC are not immortalised cells. Each attempt required a fresh CD34+ culture which, once begun, had a limited lifespan of around 3 weeks. This gave minimal

time for growth and selection of the knockdown cells. In addition, the expense of the CD34+ haematopoietic stem cells was a limiting factor in both the number of cells I could start with at the beginning of each spinoculation, and the number of attempts. The spinoculation process, occurring during the peak expansion period of the cRBC culture, also appeared to have a detrimental effect on the cell growth and Puromycin selection decreased cell numbers further (Figure 4-22). Control cells (both GFP and scrambled shRNA controls) regained some growth after selection (Figure 4-22), however CR1, GYPA and GYPC knockdown cells did not, and the resulting cell numbers by day 14-18 were too low for meaningful rosetting experiments to take place. These limitations could potentially be overcome by starting with higher cell numbers.

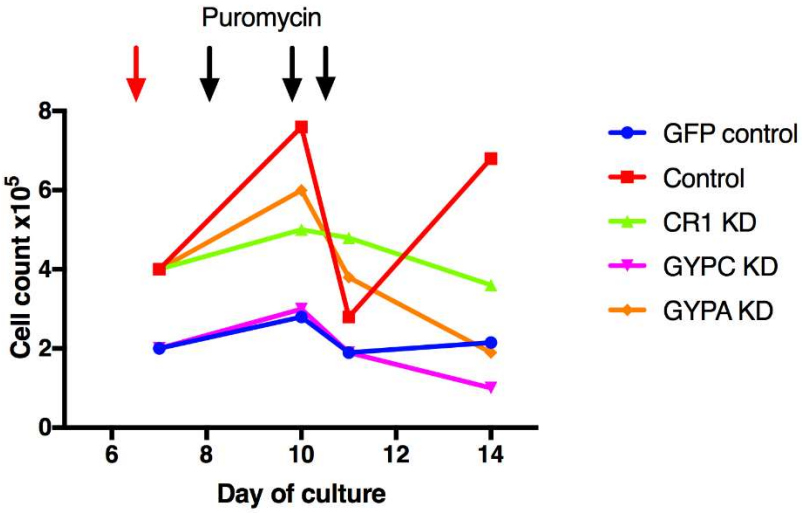


Figure 4-22 cRBC growth and lentiviral transduction on day 7. cRBC from the same culture were transduced with lentiviral particles using the spinoculation method on day 7 (red arrow). $2x10^5$ cells were transduced with the GFP control and GYPC shRNA lentiviral particles, while $4x10^5$ cells were transduced with the others. Cells were cultured as per the usual method and Puromycin was added on day 8, 10 and 11 (black arrows). On day 14, all remaining cells were used for flow cytometry experiments.

Nevertheless, the spinoculation lentiviral transduction did appear to work to a certain extent. Green fluorescent protein (GFP) was incorporated into the control cells demonstrating successful delivery of the shRNA into the cells (Figure 4-23 A). However, knockdown results were disappointing with

minimal reduction in receptor expression seen for GYPC, GYPA and CR1 shRNA transduced cells (Figure 4-23).

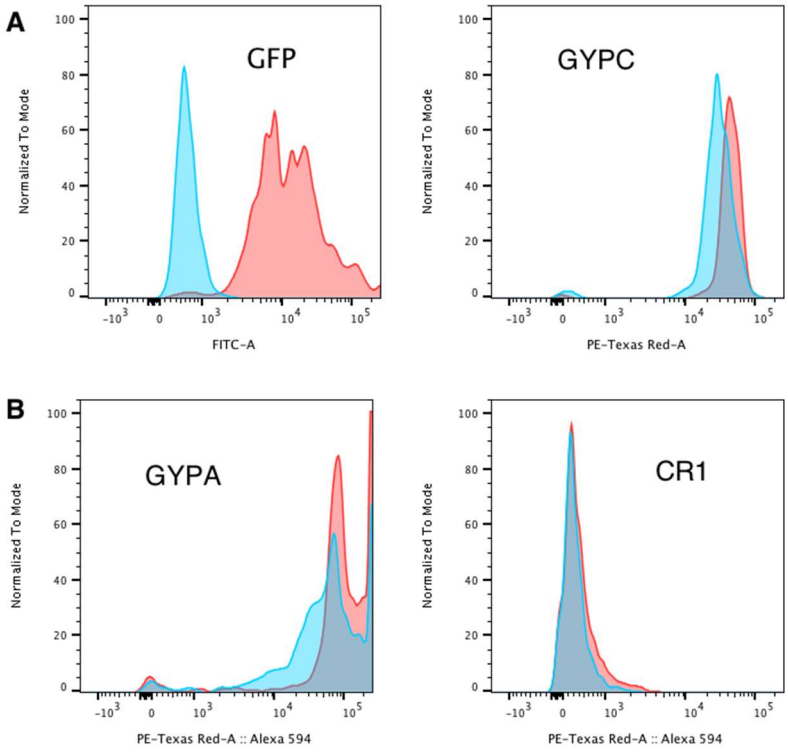


Figure 4-23 Flow cytometry plots showing GFP or receptor expression after shRNA lentiviral transduction of cRBC. cRBC were transduced on day 6/7 and results shown are from day 14. A shows GFP expression (left) or GYPC expression (right) on GFP control (RED) and GYPC shRNA transduced cells (BLUE). B shows expression of GYPA (left) or CR1 (right) on control cells (RED) and those transduced with the relevant shRNA (BLUE). Three attempts at transduction were made with similar results each time, these plots are from the third experiment

The estimated percentage efficiency of each knockdown was calculated using the formula developed by Egan *et al.* (Egan et al., 2015) which is based on median fluorescence intensity (MFI) after correction for background using the isotype control; 100-[MFI_{KDcRBC} /$MFI_{control\ cRBC}$] x100. Results are shown in Table 4-5. Of note, while the percentage knockdown for CR1 appears impressive, this is merely due to the very low expression levels in both knockdown and control (in keeping with the low CR1 expression seen on cRBC shown above), and the flow plots give a better representation of the level of knockdown. Three attempts at generating knockdown cells were

made, including the pilot experiments with different MOI (Appendix 1), with cRBC from different donors and the results were similar throughout.

Table 4-5 Numbers used to calculate the percentage knockdown, according to the formula described in (Egan et al., 2015)

Receptor	$MFI_{KD\ cRBC}$	$MFI_{KD\ cRBC}$ (after correction)	$MFI_{control\ cRBC}$	$MFI_{control\ cRBC}$ (after correction)	Percentage knockdown
GYPC	20232	19965	26828	26561	24.8%
GYPA	58179	57912	91636	91369	36.6%
CR1	277	10	321	54	81.5% (see text)

MFI= median fluorescence intensity of PE-Texas Red (Alexa 594) taken from FlowJo, after subtracting the MFI of the isotype control labelled cell (267). Gates are as shown in Appendix 1.

4.7 Discussion

4.7.1 Comparison of cRBC with mature peripheral erythrocytes

The aim of this section of my thesis was to explore the potential of generating genetically modified erythrocytes and the use of these cells in rosetting assays to help disentangle the complex mechanisms of rosetting. While iPSC initially seemed to have many of the features desirable for this type of approach, in reality the heterogenous and immature nature of the cultured iPSC suggested more work needs to be done to create an end product which is a good approximation of mature peripheral erythrocytes. The cRBCs, however, matured and enucleated to produce cells which, morphologically and on flow cytometry, resembled reticulocytes and bound just as well, if not better, to rosetting rPfEMP1 proteins. But once again, the perils of using recombinant, laboratory generated proteins only as a surrogate for infected erythrocytes was revealed, when the rosetting phenotype of cRBC, was found to be markedly reduced for parasite strains R29 and ITvar60. As discussed in Chapter 3, it is important to bear in mind that the binding of individual rPfEMP1 domains may not accurately represent the adhesion of infected erythrocytes *in vivo*, possibly due to the structure of the full-length PfEMP1 leading to masking of the binding epitopes (Resende et al., 2009).

4.7.2 A new rosetting receptor?

Initially it was somewhat disappointing to discover that the cells which seemed to be such a good proxy for mature erythrocytes did not rosette normally. However, after some consideration, I realised this represented an excellent opportunity to search for potential new rosetting receptors. My analysis using the data published by Dankwa *et al*. is certainly not a systematic screen and a more structured bioinformatic approach may well throw up more potential candidates. Nevertheless, Band 3 seemed like a good start. Band 3 is known by a number of different names; CD233, AE1,

SLC4A1 (solute carrier 4 family of bicarbonate transporters) (Kalli and Reithmeier, 2018), and consists of 911 amino acids arranged across 14 transmembrane regions as shown in Figure 4-24 and Figure 4-25 (Arakawa et al., 2015, Kalli and Reithmeier, 2018). Arakawa *et al*. (Arakawa et al., 2015) have described the crystal structure of Band 3, and Figure 4-25 taken from their paper illustrates the complex topology of Band 3 in which transmembrane segments are separated into 'core' or 'gate' domains, and sequential transmembrane segments may not be directly adjacent to each other. Band 3 is found exclusively on erythrocytes and within the renal tubules. In erythrocytes it functions as a chloride/bicarbonate exchanger enhancing the ability of blood to carry carbon dioxide in the form of bicarbonate (Kalli and Reithmeier, 2018). Each erythrocyte carries around one million Band 3 molecules which are arranged as dimers or tetramers in complex with Ankyrin, protein 4.2 and the Rhesus complex (Kalli and Reithmeier, 2018). A unique interaction with GYPA forms the Wright^{b} antigen which is located on the extracellular loop between two of the transmembrane regions (Figure 4-24). Of interest, around 50% of the total ABO blood group antigens are also carried on Band 3 (Bruce et al., 1995).

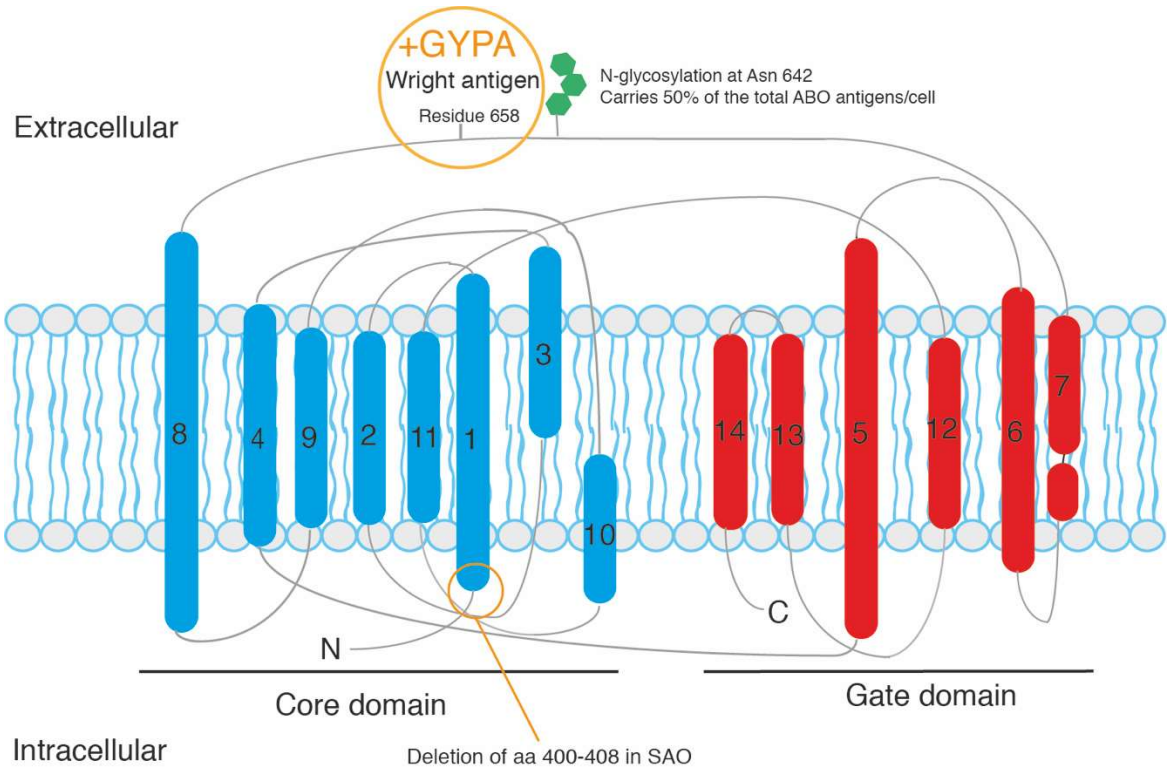


Figure 4-24 the structure of Band 3 on the erythrocyte membrane. The data used to produce this figure are from (Arakawa et al., 2015, Kalli and Reithmeier, 2018). The gate domain is the functional anion exchange part of the molecule. Red and blue coloured blocks represent the transmembrane segments of the molecule with are linked by the grey lines. SAO = south Asian ovalocytosis.

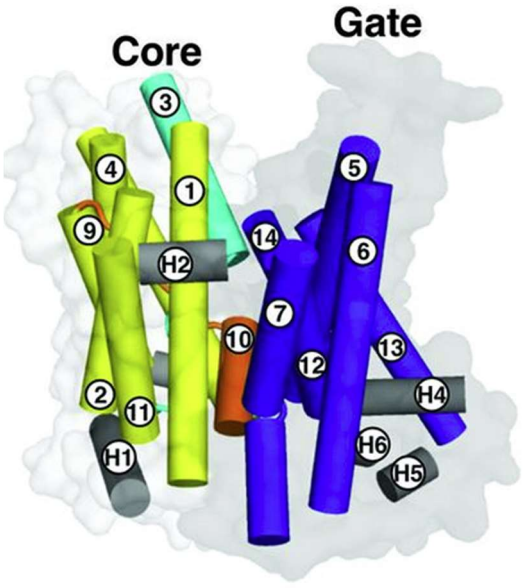


Figure 4-25 Overall structure of Band 3, viewed in the plane of the membrane. Taken directly from (Arakawa et al., 2015)

Polymorphisms in Band 3 form the antigens of the Diego blood group and a specific autosomal dominant mutation, causing the deletion of amino acids 400-408, leads to the condition South Asian Ovalocytosis (Cooling, 2015, Schofield et al., 1992). This condition, which is nearly always heterozygous, results in stiffer, oval shaped erythrocytes and has been shown to protect against both *P. vivax* and cerebral malaria in *P. falciparum*, though the exact mechanism of protection is unclear (Genton et al., 1995, Allen et al., 1999, Cooling, 2015).

Interestingly, however, my experiments with BRIC 6 Fab, (which do not react with SAO erythrocytes), failed to disrupt rosettes in most strains, while Fab of antibodies against the Wright^{b} antigen (BRIC 14) had a profound effect. These data suggest to me that SAO may not protect through the mechanism of reduced rosetting, and most significantly, it is possible that Wright^{b} is an important host rosetting receptor which will be discussed in more detail in Chapter 5. This hypothesis fits with some of the presumed features of the unknown rosetting receptors as discussed in Chapter 1, for example, Wright^{b} is resistant to Ficin/papain, trypsin, chymotrypsin, pronase and sialidase (Figueroa, 2013). However, once again, the antibody “block” method used here it unable to determine whether the rosette disruption is the result of the Fab blocking host Wright^{b} antigen, or an interaction with the infected cell. Hence the need to produce knockdown/out cells.

My attempts to do this using cRBC and lentiviral transduction of shRNA leave much to be desired. My knockdown efficiency was poor and the cell numbers remaining at day 18 were so low as to be impractical for use. Lee *et al.* (2014) and Niang *et al.* (2014) report knockdown efficiencies of 81.5% and 89-97% (for GYPC) respectively, however additional information in the supplemental data of Lee *et al*. suggests that this figures may have been the best results of multiple attempts (supplemental figure 3 from (Lee et al., 2014)). Details between my methods and those described in these papers do vary and, perhaps importantly, the shRNA lentiviral particles were different

(for example Lee *et al.* purchased Lentivector MISSION shRNA Target Set NM_002101 from Sigma Aldrich). Therefore, there is potentially much room for further optimization of my techniques. However, the challenges of using primary cells for this type of assays remain; without an immortalized cell line, the genetic manipulation must be repeated for each culture and carrying out rosetting assays within a strict timescale is a technical challenge when using multiple parasites lines. The BEL-A cells, “an immortalized adult human erythroid line” (Trakarnsanga et al., 2017), could represent a solution to this problem, however, as yet, we have been unable to obtain access to these cells. This led us to contact the Professor Duraisingh at the Harvard University T.H. Chan School of Public Health, whose laboratory has extensive experience of lentiviral shRNA transduction (Bei et al., 2010, Egan et al., 2015). While our original plan had been for me to visit their laboratory to learn how to improve my cRBC knockdowns, it transpired that Professor Duraisingh and his team had developed their own version of the BEL-A cells, the “EJ” cells, and my work with this fantastic new resource forms the next chapter of my thesis.

4.7.3 Strengths and limitations

Aside from my limited success in generating usable cRBC knockdowns, there are other important caveats to the data presented, which have already been described in the methods. The comparison of rosetting ability between the mature peripheral erythrocytes and the cRBC (Figure 4-14, Figure 4-15 and Figure 4-16) should have been made with matched peripheral erythrocytes to the bone marrow donor, however this is challenging and (if possible) very expensive when using commercial donors. In particular, the different blood groups could have an impact on rosetting, as some parasites, for example ITvar60, appears to have preference for rosetting in certain blood groups (Barragan et al., 2000b). However, for this reason, the mature peripheral cells of blood group O would be arguably be at a disadvantage as no parasites appear to prefer blood group O (Rowe et al., 1995, Rowe et al.,

2007, Rout et al., 2012). The very similar findings across four independent Caucasian donors suggests it is unlikely that specific polymorphisms present in the bone marrow, but not peripheral donors can explain the differences in rosetting ability for ITvar60 and R29, however this is not a possibility I can completely rule out.

As mentioned above, my analysis of the data provided by Dankwa *et al.* may be overly simplistic. A fuller analysis using data from (Wilson et al., 2016) is showed in Table 4-6. This table combines the results from Rowe *et al.* (1997) testing of rare red cell variants, and the ratio of blood group antigen protein levels in mature erythrocytes versus cRBC cultured from both adult and cord CD34+ cells given in Wilson *et al.* Of the blood group antigens downregulated in the cultured cells (both cord and adult), the Kell, Scianna, MNS, JMH, Colton, Dombrock and Diego stand out as the most different (highlighted in bold in Table 4-6). Of these, Rowe *et al.* tested null phenotypes of Kell, MNS, JMH and Colton and found no evidence of reduced rosetting. The Scianna and Diego polymorphisms are not completely null cells and Dombrock null cells were not testedtherefore in addition to my investigation into Band 3/Diego, perhaps the Scianna and Dombrock blood groups are worthy of further study.

Table 4-6 Comparison of data from Rowe et al. 1997 and Wilson et al. 2016, who compared blood group antigen protein levels in peripheral and cultured erythrocytes. Wilson et al. performed mass spectroscopy on pooled samples of matched peripheral erythrocytes and reticulocytes cultured from CD34+ cells derived from either adult peripheral blood or umbilical cords. In bold are the figures for which the ratio of expression in peripheral vs cRBC was notably reduced in both cord and adult cRBC

Blood group system	Antigen(s)	(Rowe et al., 1997)	Effect on rosetting	Adult RBC/cRBC (Wilson et al., 2016)	Cord RBC/cRBC (Wilson et al., 2016)
Knops	CR1	null	Yes, reduced	1.885	1.083
Kell	Kell	K_o, null	No	**3.245**	**2.189**
ABO	A, B, H antigen	Bombay, i.e. null	No		
Rh	Rhesus	Null	No	D- 2.872 C- 1.431	D- 8.832 C- 5.235
XK	Xk glycoprotein and Kell	McLeod, lacks XK gene	No	2.411	2.520
Landsteiner-Wiener	ICAM4	a-b-	No	2.414	4.418
Kidd	JK antigen	Jk-,	No	SCL14A1- 2.675 Urea transporter 1- 2.844	SCL14A1- 1.397 Urea transporter 1- 2.518
Duffy	Duffy	a-b-,	No	ND	ND
Gerbich	GYPC and GYPD	-1,-2,-3,	No	2.685	2.892
Lutheran	Lutheran, basal cell adhesion molecule	a-b-	No	2.373	1.155
Gregory	Gregory	a-	No	ND	ND
JMH	Semaphorin 7A	null	No	**5.814**	**3.986**
MNS	GYPA and GYPB	M^kM^k	No	**12.251**	**11.878**

Scianna	ERMAP	-1,-2	No	**3.127**	**2.423**
P	P antigens	Tja-, now referred to as p	No	ND	ND
Colton	Aquaporin 1	a-b-	No	**5.165**	**3.412**
Indian	I	Adult i	No	1.946	1.188
Chido	Complement C4	null	No	6.252	1.575
Lewis	Lewis	a-b-	No		
Xg	Xg(a) and CD99	null	No	2.014	0.741
Diego	Band 3	b-	No	**3.826**	**3.604**
Lan	Lan, carried on ABCB6	null	No	0.816	1.166
Vel	Vel/small integral membrane protein 1	null	No	ND	ND
Yt	Acetylcholinesterase	ND	ND	2.742	1.406
Junior	ABCG2	ND	ND	2.204	2.023
Ok	Basigin	ND	ND	0.954	1.260
CD59	MAC inhibitory protein	ND	ND	2.014	0.741
Cromer	CD55 glycoprotein	ND	ND	2.113	2.026
Dombrock	GPI fixed glycoprotein	ND	ND	**3.086**	**3.262**
RHAg	Rh- associated glycoprotein	ND	ND	1.431	5.235

ABC= human ATP-binding cassette, ERMAP= Erythrocyte membrane associated protein

The data generated by my Fab experiments is exciting, but not completely conclusive. While it appears that the Wright^{b} antigen is key, it is possible that the whole Band 3 molecule is also involved. The BRIC 6 Band 3 antibody was chosen in relation to SAO, but it is possible that its limited binding, particularly in Fab form, does not block access of the parasitized cells to the vital part of the Band 3 structure, thus rosetting is not affected. Using a panel of Band 3 antibodies directed against different epitopes could help untangle this further.

4.7.4 The way forward

More work is required to optimize the shRNA protocols to produce usable knockdown cRBC, however, since beginning this project, new cells lines have emerged, namely the BEL-A and (as yet unpublished) EJ immortalized erythroid precursors which could offer significant advantages over the cRBC system. These cells are immortalized and amenable to genetic manipulation with CRISPR/Cas9 technology, thus presenting a much more useable tool. cRBC do retain some advantages over these immortalized lines, in particular excellent enucleation, and my work using cRBC has led to a potentially very exciting discovery. However, I will now focus my final chapter on the use of EJ cells to create knockdown erythrocytes in order to further investigate the essential host rosetting receptors across different *P. falciparum* strains.

5 CHAPTER V: USING AN IMMORTALISED ERYTHROID LINE TO GENERATE CRISPR/CAS9 KNOCKOUT ERYTHROID PRECURSORS TO INVESTIGATE NEW ROSETTING RECEPTORS

5.1 Abstract

Data from rosette disruption experiments using antibody fragments suggest that the Wright^{b} antigen may represent an important novel, strain-transcending, host rosetting receptor. However, confirmation of these results is required using a different technique; the use of knockout erythrocytes. Until recently, the ability to generate useful erythrocyte knockouts has been hampered by the lack of an immortalised erythroid line. However, over the past few years, a number of different immortalised lines have been published including the HiDEP, HUDEP and BEL-A cells. Another, as yet unpublished, line has been developed by the Duraisingh laboratory at Harvard University; the EJ cells. These cells differentiate into orthochromatic erythroblasts and are amenable to genetic manipulation using CRISPR/Cas 9 technology.

In this chapter, I characterise these EJ cells in terms of morphology, receptor expression and rosetting ability and discover that, unlike the cRBC, EJ cells can form rosettes with all tested parasite lines. CR1, GYPC, Band 3 and GYPA knockout EJ cells were characterised and the rosetting of CR1, Band 3 and GYPA knockouts compared to that of wildtype EJ cells. Cells which lack the Wright^{b} antigen (Band 3 and GYPA knockouts) showed significantly reduced rosetting across all parasite lines, adding weight to the hypothesis that the Wright^{b} antigen is an important host rosetting receptor and potential therapeutic target.

5.2 Introduction

The exciting data from the Fab experiments shown in Chapter 4 suggest that the Wright^{b} antigen may represent an important, novel, strain-transcending, host rosetting receptor. However, this hypothesis would be greatly strengthened if the results could be reproduced using alternative methods, for example using knockdown or knockout erythrocytes. My attempts to generate such erythrocytes using shRNA interference with cRBC revealed the limitations of these cells, and I was keen to identify a new source of immortalized erythroid cells which would be more amenable to newer genome editing techniques such as CRISPR/Cas9.

5.2.1 Immortalized erythrocyte lines: HiDEP, HUDEP and BEL-A cells

In recent years, a number of different immortalized erythroid lines have been published (Table 5-1) including the iPSC/HiDEP and HUDEP lines (Kurita et al., 2013) and the BEL-A cells (Trakarnsanga et al., 2017). The key limitation with all of these lines appears to be poor enucleation, a vital stage of the process when generating erythrocytes suitable for blood transfusion (Anstee et al., 2012, Kurita et al., 2013, Trakarnsanga et al., 2017). However, if the cell surface receptor complement is similar to that of enucleated cells, the presence of a nucleus does not necessarily preclude the use of such cells in rosetting assays. An important potential caveat is that the shape and reduced deformability of these nucleated cells (Guzniczak et al., 2017) may impeded rosetting, however we felt this was worth investigating further.

In 2013, Kurita and colleagues published data on two immortalized erythroid lines, one from iPSC; HiDEP, and the other from CD34+ umbilical cord cells; HUDEP (Kurita et al., 2013). The HiDEP (human iPSC cell-derived erythroid progenitor) cells were produced by first transducing human iPSC with the TAL1 erythroid differentiation gene; this stage proved essential in order to produce cells which could be differentiated into haematopoietic cells (Kurita

et al., 2013). Cells were induced to differentiate into haematopoietic stem cells using erythropoietin (Grover et al., 2014), then lentivirally transduced with a tetracycline-inducible human papilloma virus 16 (HPV)-E6/E7 expression system, and cultured for around 100 days on OP9 feeder cells with stem cell factor, erythropoietin, dexamethasone and doxycycline. The E6 and E7 proteins have an oncogenic function by inhibiting p53 and retinoblastoma tumour suppressors thus promoting immortalization (Münger and Howley, 2002). To produce the HUDEP (human umbilical cord blood - derived erythroid progenitor) line, human umbilical cord CD34+ blood cells were transduced with the same tetracycline-inducible HPV-E6/E7 expression system (Kurita et al., 2013). After maturing the immortalized erythroid precursors in a similar way to that described for the EJ cells in section 2.3, both HiDEP and HUDEP cells produced functional haemoglobin, with the HUDEP-2 line producing both α and β globin compared to the γ globin of the HiDEP cells. However, enucleation levels were very low (Kurita et al., 2013). Of note, the HUDEP cells have been karyotyped and have a highly abnormal chromosomal complement with trisomy 6, 8, 19 and 21 (Vinjamur and Bauer, 2018). One of the HUDEP lines, HUDEP-2, has been genetically manipulated using CRISPR/cas9 techniques in a number of studies, mainly focusing on upregulating γ globin as a possible therapy for haemoglobinopathies such as Thalassemia (Antoniani et al., 2018, Vinjamur and Bauer, 2018, Chung et al., 2019).

The Bristol Erythroid Line-Adult (BEL-A) cells were created from adult bone marrow CD34+ cells using a tetracycline-inducible HPV-E6/E7 expression system similar to that of the HUDEP cells (Trakarnsanga et al., 2017). Unlike the HUDEP cells, however the enucleation of BEL-A cells is reportedly up to 30% (Trakarnsanga et al., 2017). Cell surface receptor patterns are similar to those of cRBC and the haemoglobin produced was predominantly adult HbA. The deformability of enucleated BEL-A cells was comparable to that of normal erythrocytes and had similar survival rates *in vivo* when injected into immunodeficient mice (Trakarnsanga et al., 2017). Trakarnsanga and

colleagues showed significant knockdown of RhAG using shRNA methods, and a subsequent study has used CRISPR technology to produce a cell line with multiple receptor knockouts including ABO, Rhesus, Kell, Duffy and Glycophorin B (Hawksworth et al., 2018).

5.2.2 The Harvard EJ cells

As the BEL-A cells are not yet available outside the developing institution, other groups have generated their own, unpublished, immortalized erythroid lines, including the Duraisingh laboratory at Harvard University, USA. In May 2018, I travelled to Boston to learn more about these cells, named the 'EJ' cells, after the postgraduate student who developed them, Dr Erik James Scully. The following information regarding the EJ cells is from personal communication with Professor Duraisingh, Dr Erik Scully, Dr Estela Shabani, Dr Becca Lee and others in the Duraisingh laboratory. The EJ cells were created by harvesting peripheral blood mononuclear cells from the blood samples of an anonymised patient with haemochromatosis. All cells were then transduced with the same HPV16 E6/E7 expression system used to generate the BEL-A and HUDEP lines, and cultured for around 60 days using erythropoietin to select for erythroid cells (Grover et al., 2014). Cells were selected by limiting dilution and cloned to form the EJ line. EJ cells are of blood group O and produce normal adult haemoglobin. In terms of morphology and receptor expression, EJ cells are approximately equivalent to day 10 of cRBC culture. Enucleation levels are not as high as reported for the BEL-A cells, ranging from 1-15%. The Duraisingh laboratory has successfully generated multiple different knockout lines using CRISPR/Cas 9 genome editing and the wildtype EJ cells used in this thesis have all been edited to express Cas9 when selected with Blasticidin (further details in the methods below). A comparison of the different immortalized erythroid lines is showed in Table 5-1. Of note, I have not considered here the immortalized erythroid lines generated from human embryonic stem cells (Ma et al., 2008) or leukaemic cells (Okuno et al., 1990).

Table 5-1 Comparison of immortalised erythroid lines. Data taken from (Kurita et al., 2013, Trakarnsanga et al., 2017) and personal communication with Duraisingh laboratory.

	Developing laboratory	Original source	Immortalization technique	Globin type	Enucle-ation
HiDEP	Nakamura, RIKEN BioResource Center, Japan	Human iPSC	Transduced with TAL1 gene then Tet-inducible HPV 16 E6/E7 expression system	α, γ and ε	Very low
HUDEP-2	Nakamura, RIKEN BioResource Center, Japan	CD34+ umbilical cord cells	Tet-inducible HPV 16 E6/E7 expression system	α and β	Very low
BEL-A	Frayne, University of Bristol, UK	CD34+ adult bone marrow cells	Tet-inducible HPV 16 E6/E7 expression system	α and β	Up to 30%
EJ	Duraisingh, Harvard University, USA	PBMC from peripheral blood of patient with haemochro-matosis	Tet-inducible HPV 16 E6/E7 expression system	α and β	1-15%

PBMC= peripheral blood mononuclear cells, Tet= Tetracycline

5.2.3 CRISPR/Cas9 genome editing

The Duraisingh laboratory had already made a number of knockout EJ cells using CRISPR/Cas9 genome editing, including knockouts for CR1, Band 3 and Glycophorin A. The CRISPR/Cas 9 method differs from RNA interference described in section 4.2.2.3 in that it creates a knock*out* at the DNA level, rather than a knock*down* by affecting mRNA (Jinek et al., 2012, Wiedenheft et al., 2012, Ran et al., 2013). Clustered regularly interspaced

short palindromic repeats (CRISPR) and CRISPR associated genes (Cas) are part of the bacterial adaptive defence system against viruses and other foreign invaders (Jinek et al., 2012). The bacterium integrates foreign DNA into its genome in between the repeats which can then be transcribed as CRISPR-derived RNA (crRNA). The crRNA complexes with Cas endonucleases, which are capable of cutting double stranded DNA, and patrols the intracellular space seeking out and destroying DNA/RNA matching the CRISPR guide sequence (Wiedenheft et al., 2012). This system can be utilized for genome editing by designing specific, single guide RNA (sgRNA), consisting of 20 nucleotides targeting the DNA of interest. The sequence must be followed by a protospacer adjacent motif (PAM) sequence; for the *S. pyogenes* system used here the PAM is 5'NGG (Jinek et al., 2012, Ran et al., 2013). The Cas endonuclease will cause a double stranded DNA break approximately 3 base pairs ahead of the PAM. This break can be repaired through either non-homologous end joining (NHEJ) or homology directed repair (HDR) (Ran et al., 2013). NHEJ tends to introduce errors during the repair which can introduce a stop codon, leading to gene knockout. HDR, on the other hand, uses a specific template and can be manipulated to introduce specific modifications (Ran et al., 2013). In this way, complete knockout/in of a specific gene of interest can be made at the level of the genome. A schematic of this process is shown in Figure 5-1.

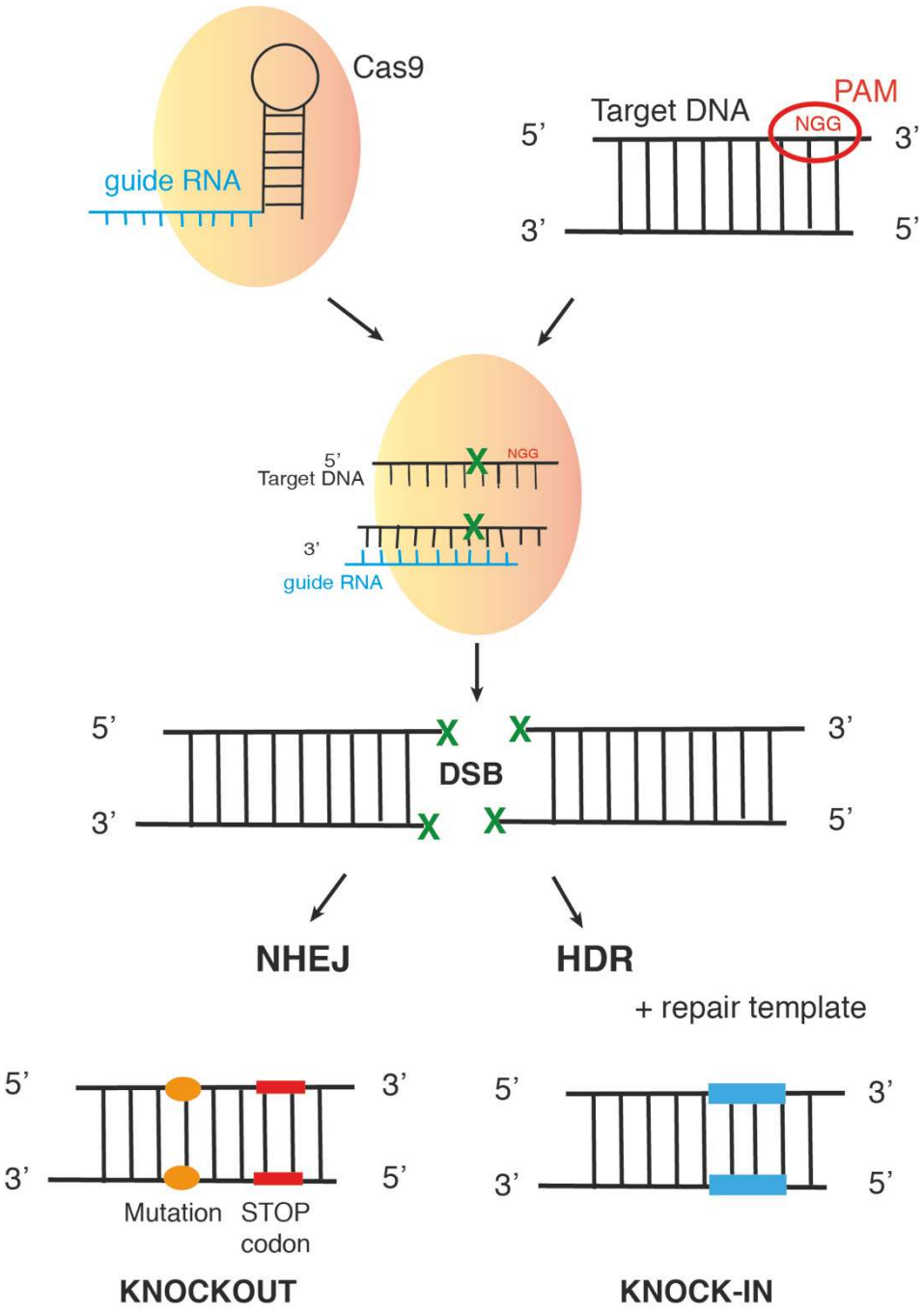


Figure 5-1 CRISPR/Cas9 genome editing. The green 'X' represents the double stranded break point. PAM= protospacer adjacent motif, DSB= double stranded break, NHEJ= non-homologous end joining, HDR= homology directed repair. Based on (Ran et al., 2013)

5.2.4 Which knockouts are of particular interest?

Having identified a potentially suitable cell line, and the technology to genetically manipulate it, the next task was to determine which knockouts

were of particular interest to test with rosetting parasites. Of the currently proposed rosetting receptors, heparan sulfate was no longer felt to be relevant given the finding in Chapter 3 that HS does not appear to be expressed on mature erythrocytes. Complement receptor 1 is relevant for some parasite lines, namely R29 (Rowe et al., 1997), and testing this receptor further with knockout erythrocytes would be useful. Glycophorin C has been investigated in a very limited fashion using knockdown cRBC and testing a knockout in a wider range of parasites could help confirm whether the findings of Lee *et al.* (2014) and Niang *et al.* (2014) can be replicated using different strains.

However, given the results of my Fab rosette disruption experiments (section 4.6.6.2), the key receptor to study was Band 3 and specifically the Wright^{b} antigen. The Wright^{b} antigen is encoded on the Band 3 gene, SLC4A1, on Chromosome 17q21-22, and results from a single nucleotide polymorphism at nucleotide 1972 (A>G) leading to Glutamic acid at residue 658 (Bruce et al., 1995). Individuals expressing the Wright^{a} antigen (with Lysine at residue 658), or those heterozygous for Wright$^{(a+b+)}$ are very rare (Bruce et al., 1995). Importantly, the Wright^{b} antigen, but not Wright^{a}, is dependent on an interaction with Glycophorin A (GYPA) for proper expression and function (Bruce et al., 1994, Bruce et al., 2004). The precise nature of this interaction is not fully understood, however it likely involves residues 61-70 of GYPA (Vengelen-Tyler et al., 1981, Ridgwell et al., 1984, Bruce et al., 1995, Huang et al., 1996). Evidence for this comes from Dantu and Miltenberger Class V (MiV) erythrocytes; both express GYPA/B hybrid molecules which lack these specific GYPA residues and do not display Wright^{b}, while another GYPA/B hybrid phenotype, St^{a} which does have this key GYPA amino acid sequence, does express Wright^{b} (Huang et al., 1996). Interestingly, the Dantu blood group was found in recent genome-wide association studies in Kenya to reduce the risk of severe malaria by 40% for heterozygotes and 70% for homozygotes (Leffler et al., 2017). There are other naturally occurring GYPA deficient cells (Table 5-2) which also completely lack Wright^{b} antigen

expression including the En(a-) (Issitt et al., 1976) and M^kM^k phenotypes (Huang et al., 1996). Of note, Rowe *et al.* (1997) had already tested M^kM^k erythrocytes and found no effect on rosetting.

Table 5-2 List of naturally occurring phenotypes which show deficient Wright^b antigen expression

	Phenotype	Comments
M^kM^k	GYPA and GYPB null	Cells apparently able to rosette (Rowe et al., 1997)
Dantu	Hybrid GYPA and GYPB	The extracellular portion of the molecule is GYPB, while the cytoplasmic domain is GYPA (Bruce et al., 1995). Associated with protection from severe malaria (Leffler et al., 2017)
En(a-)	GYPA null	Wright (a-b-) (Issitt et al., 1976, Huang et al., 1996)
MiV	Hybrid GYPA and GYPB	Extracellular portion is a hybrid of both GYPA and GYPB amino acid sequences. (Bruce et al., 1995). Very rare. Wright (a-b-) (Vengelen-Tyler et al., 1981)
Band 3 null	Band 3 null but do express GYPA	Generally not compatible with life. Few reported surviving cases (Ribeiro et al., 2000, Perrotta et al., 2005, Toye et al., 2008)

While people with a GYPA null phenotype appear to suffer no major adverse clinical consequences (unlike Band 3 null phenotypes), there is evidence to suggest GYPA does have a role in Band 3 function (Bruce et al., 2004). Bruce *et al*. (2004) have shown that anion transport is lower in M^kM^k and MiV cells and trafficking of Band 3 to the cell surface is also reduced in the absence of GYPA (Young et al., 2000). A recently published computational analysis suggests that GYPA promotes clustering of Band 3 in the erythrocytes membrane (Figure 5-2) (Kalli and Reithmeier, 2018).

Conversely, the complete lack of Band 3 is usually fatal (Ribeiro et al., 2000, Perrotta et al., 2005, Toye et al., 2008).

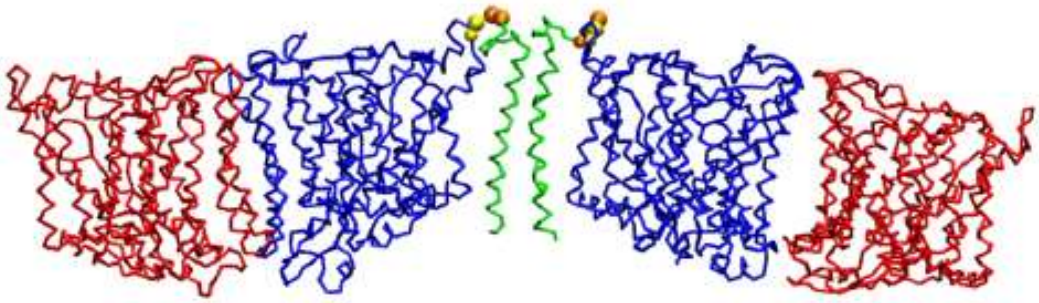


Figure 5-2 A computerized simulation model of the interaction between GYPA and Band 3, taken directly from (Kalli and Reithmeier, 2018). Band 3 monomers are shown in blue and red, while the GYPA is in green. The yellow spheres represent the GYPA Arg61 residue which is thought to be key for the interaction and the Band 3 Glu658 residue is shown in orange.

Therefore, both Band 3 and GYPA knockout cells will lack proper expression of the Wright^{b} antigen. Individually, it is not possible to dissect whether any change in rosetting in these cells is due to the lack of the Band 3 or GYPA, or the Wright^{b} antigen. However a comparison of the rosetting abilities of these cells could help clarify the essential rosetting receptor further. Both knockouts, along with a CR1 Knockout line, had already been created by the Duraisingh laboratory and were available for testing, however a GYPC knockout had not yet been generated.

5.3 Hypothesis and aims

Based on the results of the rosette disruption assays shown in Chapter 4, I hypothesised that the Wright^{b} antigen is a key, strain-transcending host rosetting receptor for *P. falciparum*. The aim of the current chapter is to confirm this hypothesis using knockout erythroid cells generated from the EJ immortalized erythroid cell line.

Firstly, I intended to culture the EJ cells for the first-time outside the developing laboratory in Harvard, then characterise the wildtype and

knockout EJ cells in terms of their morphology and cell surface receptor expression. Following this, I aimed to test the rosetting ability of both wildtype and knockout matured EJ cells to determine which receptors are essential for rosetting across a panel of diverse *P. falciparum* strains.

5.4 Methods

Details of the culture methods for maintenance and differentiation of EJ cells are included in section 2.3.

5.4.1 Characterisation of undifferentiated and mature EJ cells

EJ cells were investigated for morphology and cell surface receptor expression in much the same way as cRBCs described in section 4.4. Modifications to the techniques are described below.

5.4.1.1 Morphology by cytospin

Cytospin slides were prepared in an identical way to that described in section 4.4.2, however instead of straightforward staining with Giemsa, slides were first stained for 3 minutes with 10% May-Grünwald stain (VWR chemicals, 10047018, made up with dH_2O) rinsed in water for one minute, then stained with 10% Giemsa (filtered and made up with Giemsa buffer) for 15 minutes.

5.4.1.2 Flow cytometry for cell surface receptor expression

Preparation of cells for flow cytometry was identical to that described in section 4.4.3, including Heparinase III pre-treatment when looking for the HS stub molecules. However, the list of receptors investigated was curated to those of particular interest and a number of primary antibodies to different parts of the Band 3 antigen were included. The Band 3 antibodies used are

included in Table 5-3. Initially, BRIC 6 and BRIC 14 were of particular interest as BRIC 6 fails to react with erythrocytes with the South Asian ovalocytosis mutation, and BRIC 14 is specific for the Wright^{b} antigen. However other antibodies directed against other Band 3 epitopes were also used in later experiments. All were purchased from the International Blood Group Reference Laboratory in Bristol, UK, and were used at 14µg/ml. Isotype controls for the IgG1 antibodies were as described in Table 4-3, for BRIC 6 an IgG3 isotype control was used (mouse IgG3k, clone MG3-35, Abcam, ab18392) and for BRIC14, a mouse IgG2a isotype control was used (mouse IgG2a, Clone MOPC-173, Biolegend, 400202).

Table 5-3 List of Band 3 antibodies used in flow cytometry experiments on EJ cells, including their presumed epitopes. Data taken from the data-sheets provided by IBGRL.

Clone	IBGRL code	Species/ Class/subclass	Epitope	Other
BRIC 6	9439PA	Mouse IgG3κ	Probably extracellular loop between aa 545-567	Impedes binding of Wright^{b} antibody (Pasvol et al., 1989). Fails to react with SA ovalocytosis CD233 (Groves et al., 1983).
BRIC 14	9413PA	Mouse IgG2a,κ	Wright^{b}, aa sequence at residue 658 (GLU) of Band 3, on 4th extracellular loop connecting TM domains 7 and 8 (Kalli and Reithmeier, 2018)	Has been reported as inhibiting *P. falciparum* invasion (Pasvol et al., 1989)
BRIC 200	9468PA	Mouse IgG1κ	Probably extracellular loop between aa 545-567	
BRIC 71	9472PA	Mouse IgG1κ	3rd extracellular loop presumed to be different to others	
BRAC 17	9451PA	Rat IgG2b,κ	Probably extracellular loop between aa 545-567	Not used in this thesis

IBGRL = International Blood Group Reference Laboratory

5.4.2 Rosetting assays with undifferentiated and mature wildtype EJ cells

Rosetting assays with EJ cells were carried out as described in section 2.6.3.

Pilot tests using ITvar60 and 11019 were conducted while visiting the Duraisingh lab in Harvard University and involved testing undifferentiated and matured EJ cells. As ethidium bromide was not available, Vybrant® DyeCycle™ Violet stain (Thermo Scientific, V35003) was used to stain parasites by centrifuging the required packed cell volume of parasite culture, resuspending in 10ml of incomplete RPMI and adding 1µl of the dye (to give 1:10,000 dilution) followed by a 30 minute incubation. The parasites were then washed and MACS purified for use in the rosetting assay. For these pilot experiments, the EJ cells were stained with Vybrant® DyeCycle™ Green (Thermo Scientific, V35004) in the same manner. Otherwise, the methods were as previously described in section 2.6.3.

On my return to Edinburgh, these experiments were repeated for matured EJ cells with both the lines previously tested and for R29, 9605, HB3 and TM284. Parasites were stained with ethidium bromide and EJ cells with CellTrace™ as detailed in section 2.6.3. Again, for practical reasons, the 'control' mature erythrocytes were not matched to the original donor from whom the EJ cells were derived.

5.4.3 Lentiviral transduction and CRISPR/Cas9 gene editing of EJ cells to generate knockout cells

The Duraisingh laboratory in Harvard had already generated a number of knockout EJ cell lines, including GYPA, CR1 and Band 3, however these had not yet been characterised by flow cytometry. While I was visiting, I worked under the supervision of Dr Becca Lee to produce a GYPC knockout line. This line and the GYPA, CR1 and Band 3 knockouts were shipped to

Edinburgh on dry ice in late 2018. The methods used to generate the GYPC knockout line are detailed below.

The LentiGuide-Puro two vector system derived from *Streptococcus pyogenes* was used (Figure 5-3) (Sanjana et al., 2014). This is a two-stage approach in which cells are first transduced with a lentiCas9-Blast and selected using Blasticidin which integrates Cas9 into the cell line (GeCKO lentiviral CRISPR toolbox (Sanjana et al., 2014, Shalem et al., 2014). This was done prior to my arrival in the Duraisingh lab.

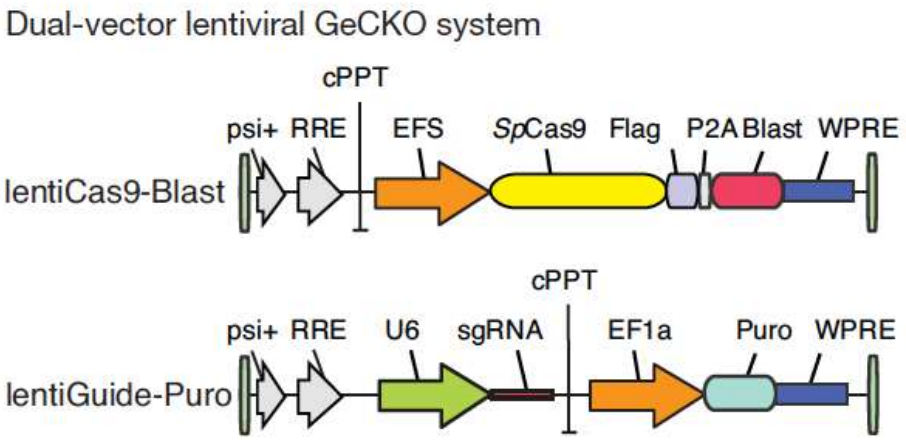


Figure 5-3 The two stage LentiGuide-Puro two vector system. Figure taken from (Sanjana et al., 2014). psi+= Psi packaging signal, RRE= Rev response element, cPPT= central polypurine tract, EFS= elongation factor 1α short promotor, Flag= Flag octopeptide tag, P2A= 2A self-cleaving peptide, Puro= puromycin selection marker, Blast= Blastocidin selection marker, WRPE= post-transcriptional regulatory element, EF1a= elongation factor 1a promotor.

The next stage (detailed below and Figure 5-4) is the introduction of a LentiGuide-Puro vector containing the guide RNA of interest, which is then selected for using Puromycin.

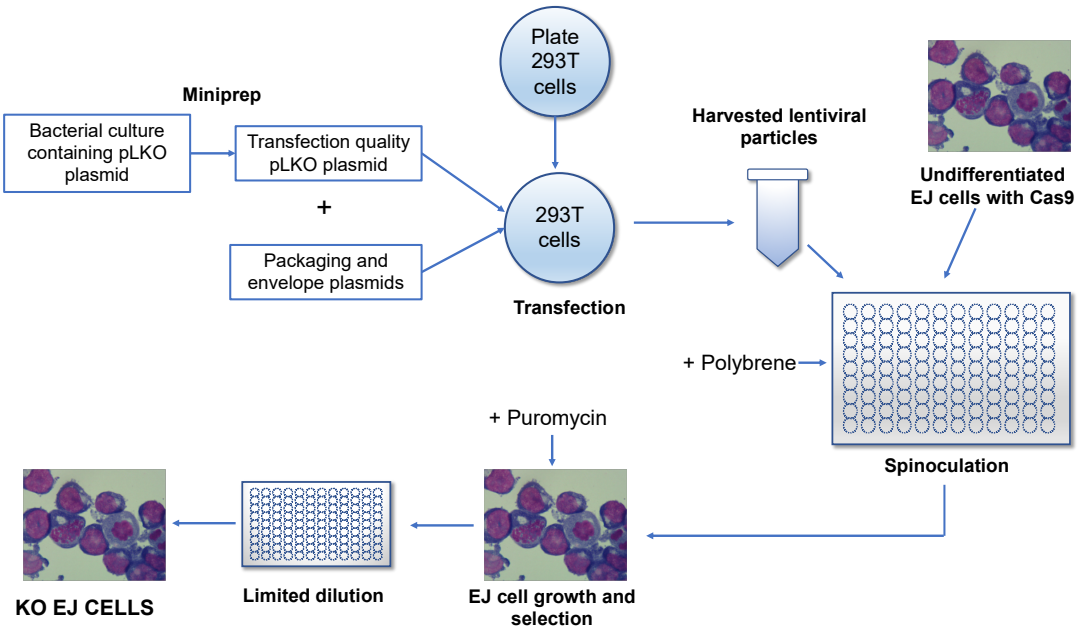


Figure 5-4 Schematic showing the production of KO EJ cells using the lentiGuide-Puro two vector system. pLKO= the CRISPR sgRNA plasmid designed to knockout the gene of interest

5.4.3.1 Production of lentiviral particles

The protocol for the production of lentiviral stocks from CRISPR plasmids was taken from The RNAi Consortium (TRC) laboratory protocols at the Broad institute (available from https://portals.broadinstitute.org/gpp/public/resources/protocols), with modifications by Dr Estela Shabani of the Duraisingh laboratory.

Packaging 293T cells were seeded at $2x10^6$ cells per 6cm Petri dish in 6 ml of seeding media (Table 5-4) giving a density of $1x10^5$ cells/cm^2 and incubated for 24 hours at 37°C in 5% CO_2. After 24 hours the cells were around 70-80% confluent and ready for transfection.

Table 5-4 Media for 293T packaging cells

Type	Media	Serum	Antibiotics
Seeding/ Maintenance media	DMEM Mediatech 10-013-CV	10% HI FBS, HyClone SH30071.03	None
Viral harvest media	DMEM	30% HI FBS	1% v/v Pen/Strep, Mediatech, 30-002-CI

DMEM= Dulbecco's Modification of Eagle's Medium, Pen/strep= Penicillin/Streptomycin, FBS= fetal bovine serum, HI= heat inactivated

The packaging (pCMV-R8.74psPAX2) and envelope plasmids (VSV-G/pMD2.G), required to encode the viral structural proteins and enzymes, had previously been obtained from the Broad institute and prepared by Dr Estela Shabani. The pLKO vector plasmid containing the GYPC targeted sgRNA (sequence AATGGTGGTAGTATGCATTG), was designed and synthesized by the Broad institute and transformed into Stbl3 bacteria which was cultured overnight from frozen plates. Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions; the bacterial culture was lysed, centrifuged and the supernatant passed through the QIAprep 2.0 spin column binding the plasmid DNA. The columns were then washed and eluted to obtain the plasmid DNA.

The transfection mixture was made by first diluting 6µl of TransIT-LT1 transfection reagent (Mirus Bio, MIR 2300/5/6) with 84µl of OPTI-MEM™ serum-free media (Invitrogen, 31985-070) dropwise with gentle mixing. This was incubated for 5 minutes at room temperature then added to the three plasmid mix (900ng of packaging plasmid, 100ng of envelope plasmid, and 1µg of pLKO vector per 6cm petri dish), again dropwise with gentle swirling and finally mixing by inversion 5-7 times. The mixture was incubated at room temperature for 30 minutes then transferred to the 293T packaging cells. The cells were incubated for 18 hours at 37°C in 5% CO_2, then the media changed to viral harvest media (Table 5-4) and the cells incubated for a further 24 hours. After this, the media containing the lentivirus was collected

and centrifuged for 5 minutes to pellet any remaining 293T cells. The resulting supernatant was stored at -80^{o}C.

5.4.3.2 Lentiviral transduction of Cas9 EJ cells

Undifferentiated EJ cells with integrated Cas9 were transduced with the lentiviral particles prepared as above using a similar spinoculation method to that described in section 4.4.7 for cRBC. One hundred µl per well of PBS/1% BSA was pipetted into a 96-well plate and left at room temperature for 20 minutes. Sufficient volume of undifferentiated EJ cell culture to give 5x10^{4} cells per transduction was centrifuged at 200g for 12-15 minutes and resuspended in pIMDM without serum (Table 2-4) at approximately 5x10^{5} cells/ml. The PBS/1% BSA block was removed from the 96 well plate and 100µl of the resuspended cells pipetted per well (5x10^{4} cells/well). A further 20µl of pIMDM was then added along with 80µl of the lentiviral mixture (estimated to give a MOI of 0.5) and 8µg of polybrene (Santa Cruz Biotechnology, sc-134220, diluted to 1mg/ml with PBS). The plate was then centrifuged for two hours at 1000g. After centrifugation, the contents of each well were transferred to a 24 well plate to which was added 2ml of complete EJ media with additives, excluding puromycin (Table 2-5). After 24 hours, 2µg/ml of Puromycin was added and from day 3 after transduction, the usual EJ maintenance protocol was followed with the addition of Puromycin at each media change.

5.4.3.3 Post transduction

After transduction, the EJ cells were maintained with Puromycin selection for 2- 4 weeks then cloned by limiting dilution. As my visit to the Harvard laboratory was for 4 weeks only, this step was carried out by Dr Becca Lee. Clones were then Sanger sequenced by the Duraisingh laboratory. The selected GYPC KO line was shipped to Edinburgh on dry ice at a later date.

5.4.4 Characterization of knockout EJ cells

Knockout EJ cells were characterised as regards their morphology and cell surface receptor expression as described in sections 5.4.1.1 and 5.4.1.2.

5.4.5 Rosetting assays with knockout EJ cells

The rosetting assays with the knockout EJ cells were conducted as described in section 2.6.3. Wildtype and knockout EJ cells were matured in parallel until day 8-10 when they were harvested for use in the rosetting experiments with MACS purified parasites (section 2.6.3). Each experiment also included a separate sample of mature peripheral erythrocytes from Scottish donors as a comparator and to ensure that the parasites were retaining their ability to rosette after MACS purification. Though the experiments were blinded, it should be noted that it was fairly easy to unintentionally determine between EJ cells and mature peripheral erythrocytes under the microscope as the vast majority of EJ cells still contained a nucleus, although it was not possible to differentiate between wildtype and knockout EJ cells. Wildtype and CR1 knockout EJ experiments were carried out separately to the wildtype, GYPA and Band 3 knockout experiments. For each parasite line, three independent experiments were carried out for each knockout line (i.e. different MACs purifications and different EJ samples), though some of the repeats were from the same maturation cycle of the EJ cells (e.g. tested on D8 and D9). Due to limited cell numbers and time constraints, parasite line 9605 was not tested with the Band 3 KO EJ cells, and the results for the Band 3 KO with parasite line 11019 are those carried out while in the Duraisingh laboratory.

5.5 Results

5.5.1 Expansion, differentiation and maturation of EJ cells

After a variable period of recovery after thawing (ranging from 1-5 weeks), undifferentiated EJ cells tripled in number on average every 2-3 days, though day to day growth did vary. In a similar way to my cRBC and the HiDEP/HUDEP and BEL-A cells (Kurita et al., 2013, Trakarnsanga et al., 2017), a significant number of cells were lost in the later stages of differentiation, however the cells did continue to divide and grow until around day 4 of the differentiation protocol. Initially, part of this cell loss was due to EJ cells adhering to the stromal cells and being left behind on day 7 when both the media and stroma were changed (see Figure 5-5), however this was improved by gently pipetting the EJ-containing spent media over the used stromal layer 2-3 times to collect adherent EJ cells before centrifugation and changing of the media.

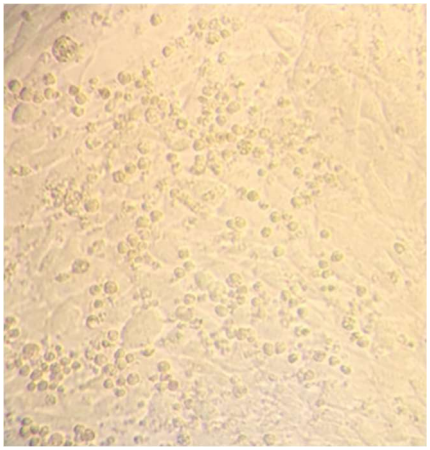


Figure 5-5 EJ cells adherent to MS-5 stroma cells after removal of the cell culture medium. Cells visualised using an Olympus CK2 inverted microscope and the x20 objective. Photograph taken with an iPhone 5 camera.

As with the cRBC, the EJ cell morphology matured with differentiation (Figure 5-6), however enucleation was very poor, around 1-3%, even when the differentiation protocol was extended to 12 days.

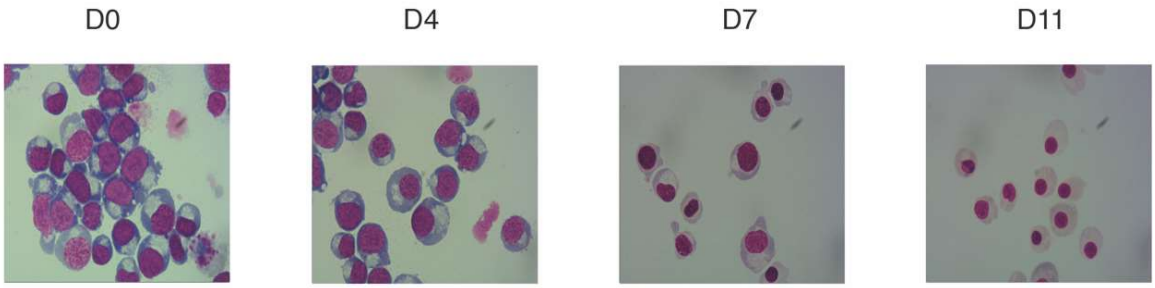


Figure 5-6 Evolving morphology of EJ cells following the differentiation protocol. Wildtype EJ cells were prepared using cytospin and May-Grünwald staining. Cells were visualized using the x100 objective on an Olympus BX40 microscope and Olympus microscope camera.

A modified differentiation protocol, without the use of supporting stromal cells, was also tried. This protocol is simpler and only requires media change on day 3 (plate at $5x10^5$ cell/ml pIMDM with 5% serum and 3IU/ml erythropoietin) and day 5 (plate at $5x10^5$ cell/ml pIMDM with 5% serum and 0.3IU/ml erythropoietin) with harvesting of cells at day 8. While this avoided the need to co-culture with stromal cells, I found a significantly higher level of cell debris from dead and dying cells was present in the later stages of the no stroma cultures and cells appeared less healthy, therefore it was decided to continue using stromal cells (Figure 5-7).

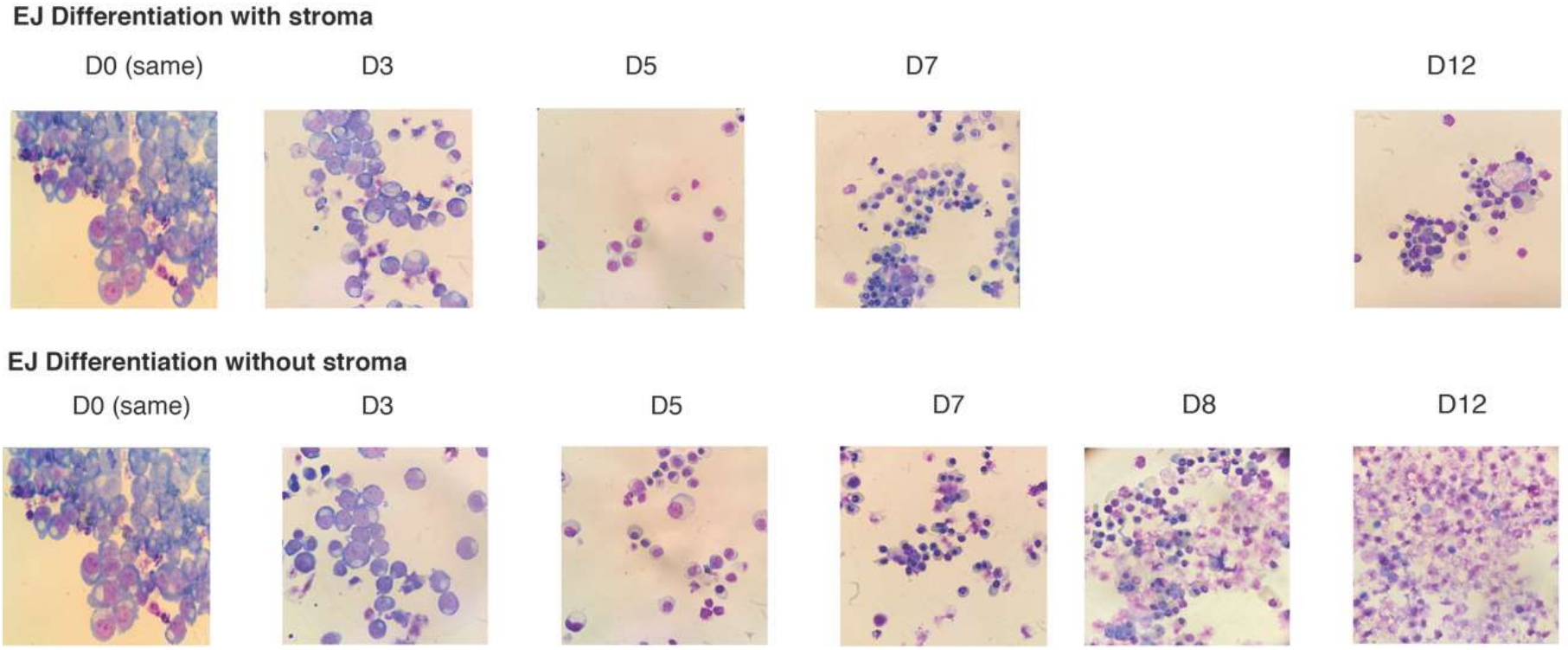


Figure 5-7 A comparison of the morphology of maturing EJ cells cultured with and without stromal support. The cells shown are CR1 KO EJ cells as these were the first EJ available for use. Cultures without stroma had significantly more debris and increased cell death as the culture time was extended.

5.5.2 Cell surface expression of undifferentiated and mature EJ cells

The cell surface expression of receptors of interest was investigated using flow cytometry. A significant amount of non-specific binding was seen as demonstrated in the IgG1 isotype control cells shown in Figure 5-8. Both the IgG/M secondary only samples and isotype controls showed high levels of background fluorescence, particularly the IgM secondary control. Despite this, specific signals could be seen above the background staining, and the overall receptor profile was similar to that of D8-10 cRBC cells (Figure 5-9, results for 3G10 have been shown in Figure 3-16).

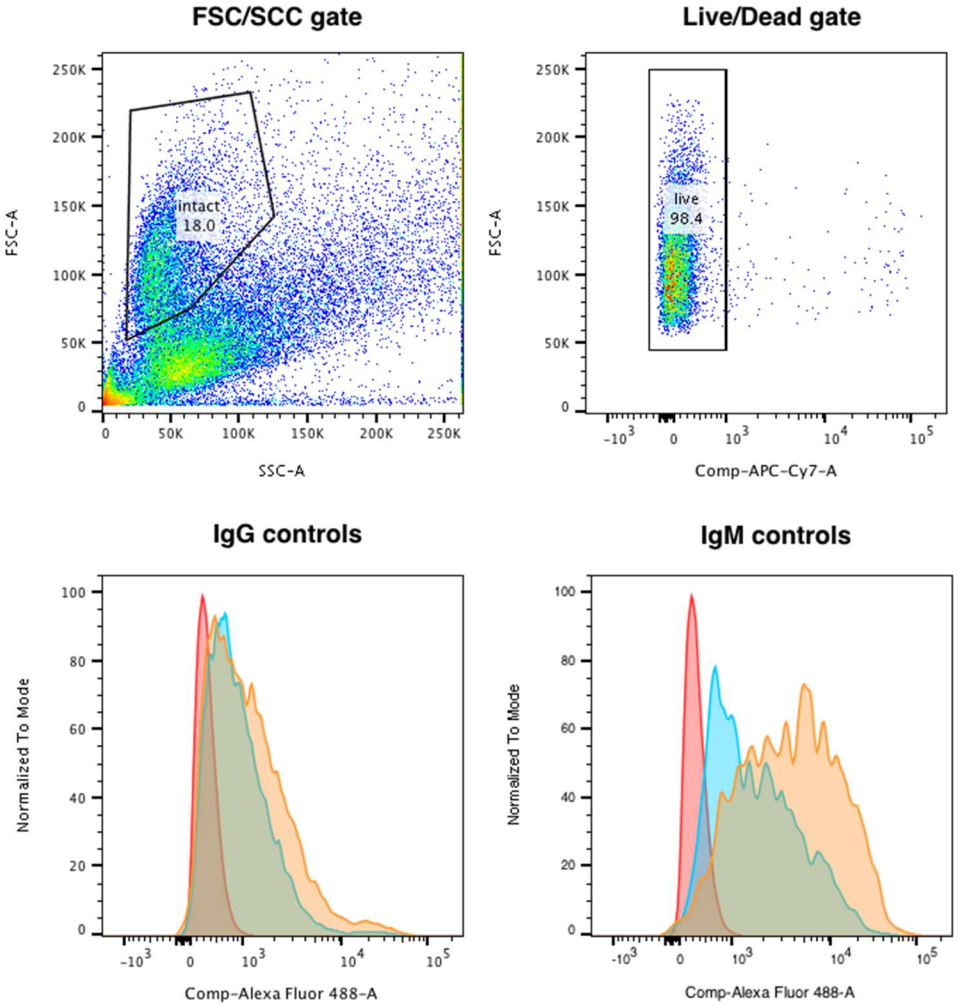


Figure 5-8 Undifferentiated EJ cells. Upper panels show flow cytometry plots demonstrating the FSC and SSC profile and gating strategies for the receptor expression profiles. Lower panels show "no antibody" samples (RED), IgG/M secondary antibody only (BLUE) and isotype control (ORANGE), demonstrating non-specific binding. IgG1 controls are on the left and IgM on the right.

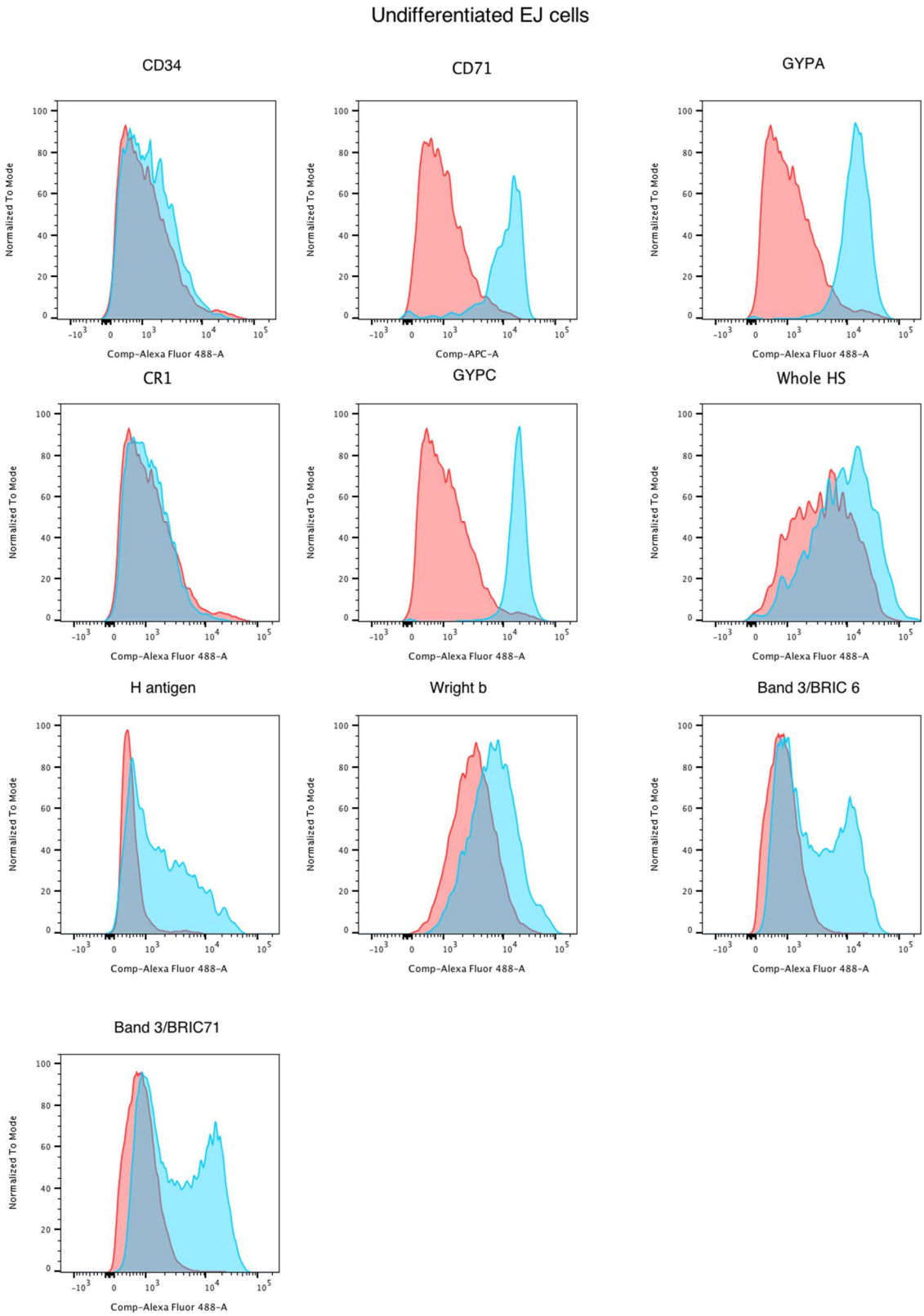


Figure 5-9 Cell surface receptor expression on undifferentiated wildtype EJ cells. RED- isotype control (varies by receptor), BLUE- receptor. All plots have been normalised to mode. Note higher background fluorescence for Wright^{b} antibody

As the cells reached the end of the maturation process, three broad populations emerged; intact nucleated, enucleated, and disintegrating cells (Figure 5-10). After excluding the large proportion of dead cells and debris, gating based on nuclear staining revealed a very small number of enucleated cells, a population of intact, mature, nucleated EJ cells, and a population of brightly fluorescing, false-positive cells (Figure 5-10A). This third population represents cells which are disintegrating but not yet dead and can be also seen clearly by IFA (Figure 5-10B). The disintegrating cells could also be defined by a lower FSC and wide-ranging SSC (Figure 5-10C), and based on this, the final intact/live gating strategy shown in Figure 5-10C was used.

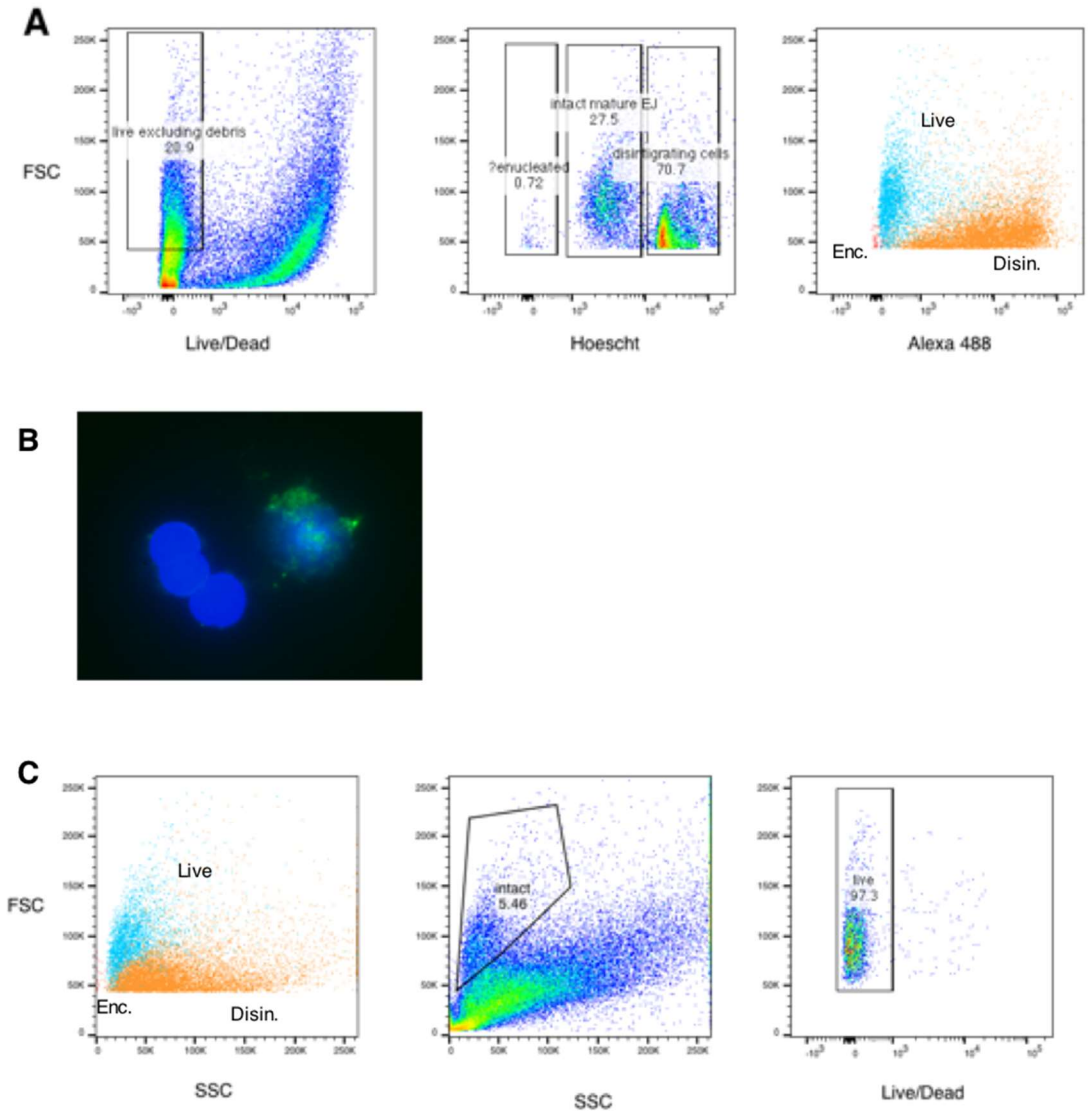


Figure 5-10 Flow cytometry profile of mature (D8) EJ cells. Cells shown are of the IgG1 isotype control samples, therefore positive fluorescence represents non-specific binding. A Far left- gating to exclude dead cells and debris based on FSC and Live/Dead stain. Centre- the three populations of cells described in the text based on FSC and nuclear staining with Hoechst. Far right- the FSC/Alexa488 profiles of each of the cell populations, demonstrating the false-positive Alexa488 signal of the disintegrating cell population. LIGHT BLUE= live, intact nucleated EJ cells (live), RED = enucleated cells (Enc), ORANGE = disintegrating cells (Disin). B IFA image of three intact and one disintegrating, false positive cell. Taken using a Yenway microscope camera with the x100 objective on a Leica DM LB2 florescence microscope. BLUE= Hoechst nuclear stain, GREEN= Alexa 488. C demonstrates how the disintegrating cells can be gated out from the start using FSC and SSC. The final gates used to determine receptor expression (by FSC/SSC followed by Live/dead staining) are shown in the middle and left plots of C.

The receptor profile based on gating for intact, live mature wildtype EJ cells is shown in Figure 5-11, the main difference from the undifferentiated cells being an increase in Wright^{b} expression and loss of HS. CR1 expression

remained very low, as seen with the cRBC (section 4.6.2); again, this could be due to low CR1 phenotype in the original donor, or a reflection of the immature nature of the EJ cells.

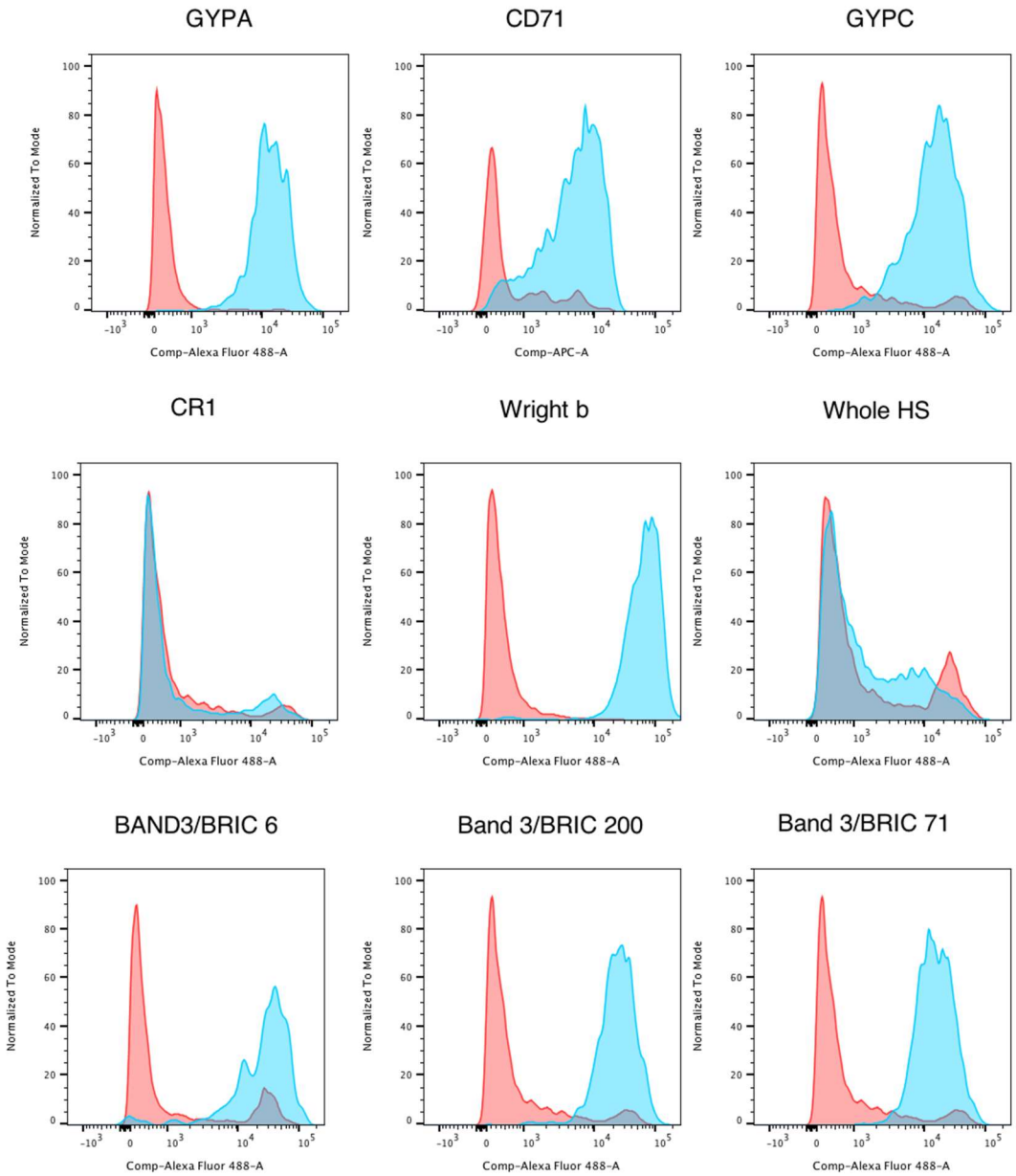


Figure 5-11 Receptor profile of mature EJ cells (D8/9 of culture) by flow cytometry. RED= isotype control (varies by antibody), BLUE= receptor.

5.5.3 Differentiated EJ cells are able to rosette with *P. falciparum* infected erythrocytes

Mature wildtype EJ cells were capable of forming rosettes with the six tested parasite lines, however immature EJ cells were not. Examples of rosettes with ITvar60 and 11019 for mature and undifferentiated EJ cells are shown in Figure 5-12. The rosette frequency of mature EJ cells was comparable to that of unmatched peripheral erythrocytes (Figure 5-18, Figure 5-19, Figure 5-20).

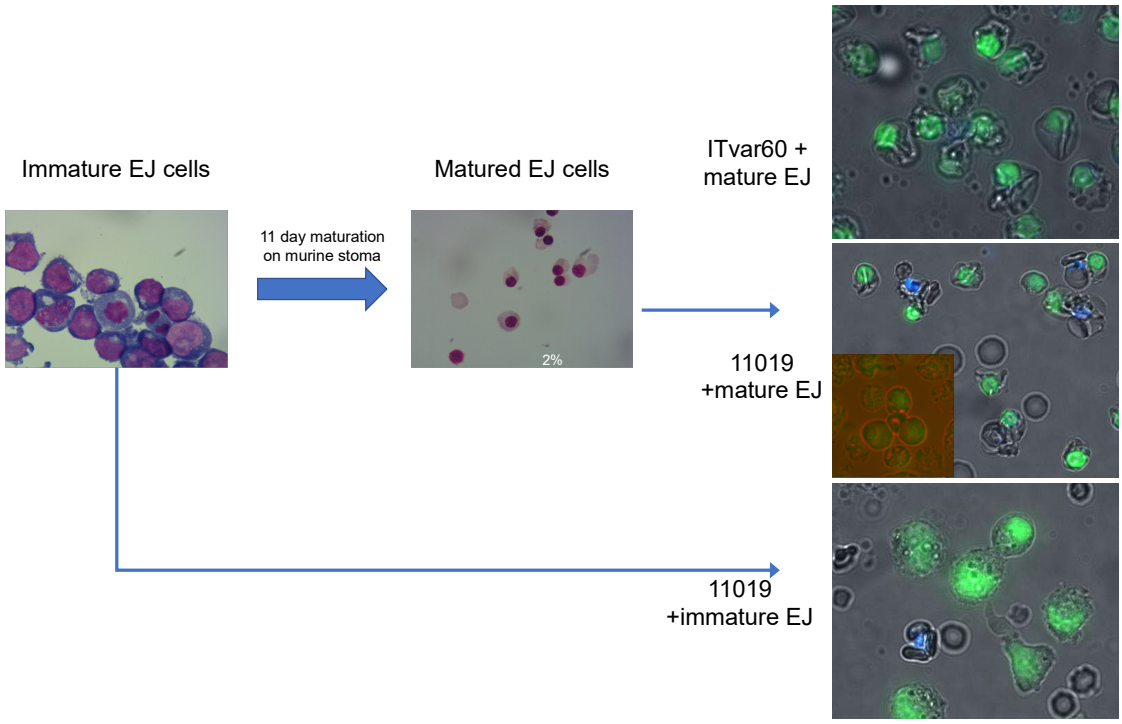


Figure 5-12 Mature EJ cells are able to form rosettes. Example images of rosette formation with mature, but not immature EJ cells with the parasite lines ITvar60 and 11019. EJ cells are stained green, infected cells are blue and unstained cells are peripheral erythrocytes. The three larger images were taken using a photometric evolve camera and the x60 objective on the Zeiss Imager fluorescent microscope at Harvard. The inset image for 11019 was taken using with x100 objective using a Yenway microscope camera on a Leica DM LB2 florescent microscope. In this image, the parasites are stained orange with ethidium bromide.

5.5.4 Morphology and receptor expression of knockout EJ cells

The morphology of the knockout EJ cells matured in a similar way to the wildtype cells (Figure 5-13). Some differences were noted including a more irregular outline and increase vacuoles within the cytoplasm seen in the Band

3 KO cells. This is unsurprising as Band 3 has an important structural role within erythrocytes.

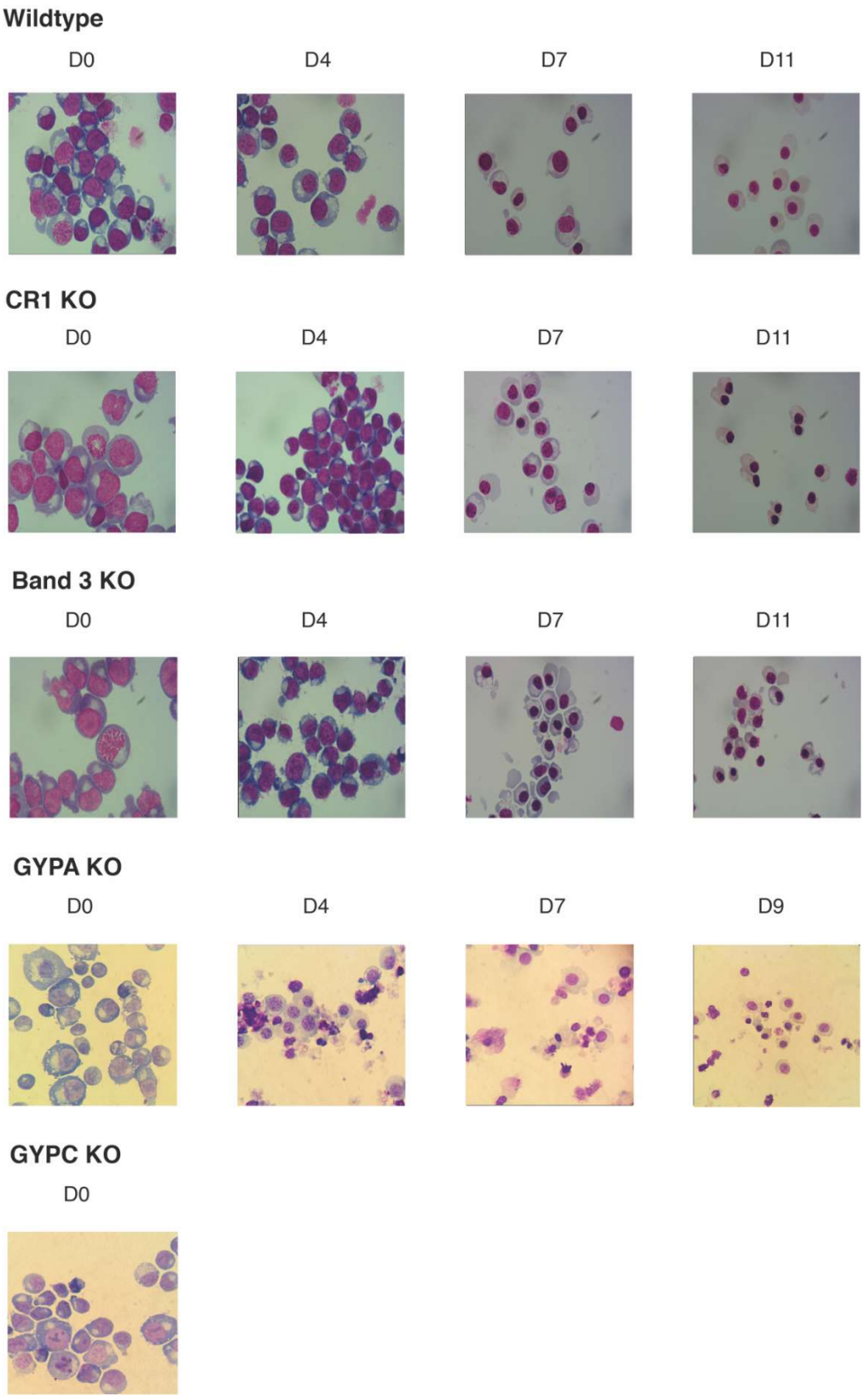


Figure 5-13 Giemsa/May Grunwald staining of maturing Wildtype and KO EJ cells. Wildtype, CR1 and Band 3 KO images were taken using the x100 objective on an Olympus BX40 microscope and Olympus microscope camera. GYPA KO and GYPC KO images were taken using the x100 objective on an Olympus BX45 light microscope and iPhone 5 camera. The GYPC cells have not yet been matured (see below).

5.5.4.1 GYPC KO EJ cells

The GYPC KO cells generated as described in the methods were tested for receptor expression in the undifferentiated state. Sanger sequencing conducted by the Duraisingh lab had indicated that a stop codon had been introduced into the GYPC gene suggesting that these cells were genetically GYPC knockouts (Dr Becca Lee, personal communication). However, on thawing in Edinburgh and prior to selection, the EJ cells were found to express GYPC on the cell surface by flow cytometry (Figure 5-14). After selection with Puromycin, GYPC expression did reduce further, but did not disappear completely suggesting that these cells may be heterozygous for GYPC expression, rather than a complete knockout. For this reason, along with the minimal rosette disrupting effect of the GYPC Fab shown in section 4.6.6.2, it was decided to concentrate on the other EJ KO for rosetting experiments.

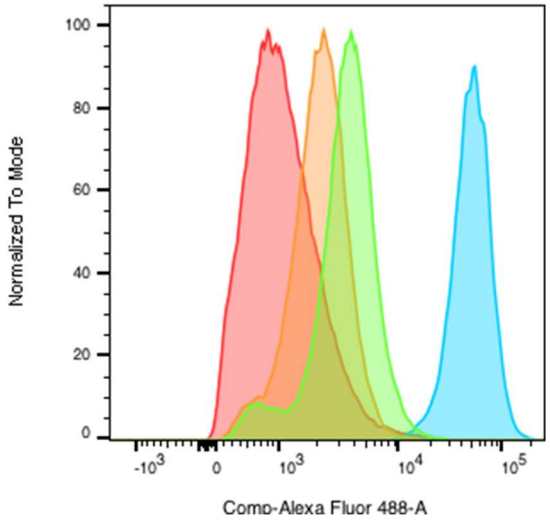


Figure 5-14 GYPC expression on immature selected and unselected GYPC and CR1 KO cells. RED- IgG isotype control primary antibody, ORANGE- puromycin selected GYPC KO cells with anti-GYPC, GREEN- unselected GYPC KO cells with anti-GYPC, BLUE- CR1 KO with anti-GYPC (WT cells were unavailable at this point). Gates are as described in Figure 5-8.

5.5.4.2 CR1 KO EJ cells

The CR1 KO EJ cell receptor profile was very similar to that of the wildtype EJ cells, which expressed very little CR1 even in an unmodified state. By flow cytometry and IFA, no obvious difference could be seen in CR1 expression between the two cell types (Figure 5-15).

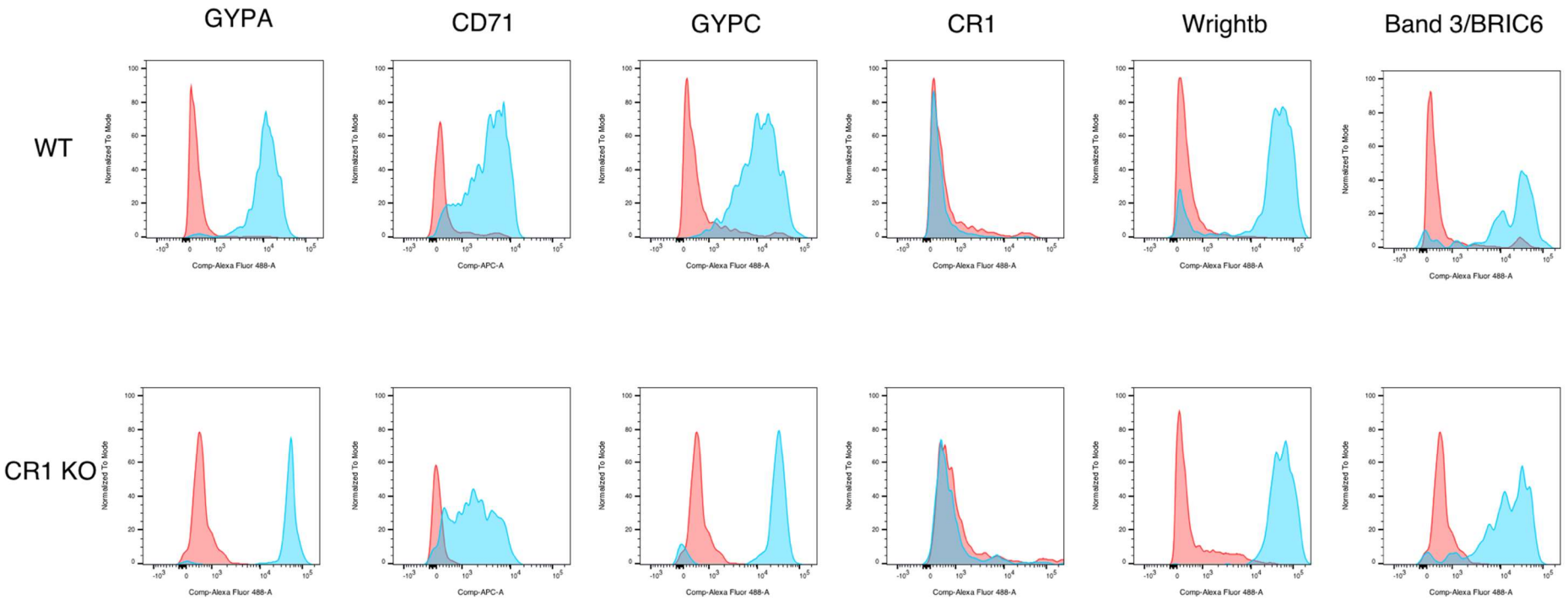


Figure 5-15 Receptor profile for mature wildtype and CR1 KO cells. Gating strategy as described Figure 5-10. All plots normalized to mode. RED- isotype control, BLUE- receptor.

5.5.4.3 GYPA and Band 3 KO EJ cells

In contrast to the GYPC KO EJ cells, both the GYPA KO and Band 3 KO did not appear to express any GYPA/Band 3 respectively when tested by flow cytometry (Figure 5-16). As expected, the Band 3 KO cells also lacked the Wright^b antigen, however in the GYPA KO, some binding of Wright^b antibody did occur, though it was reduced compared to wildtype cells. The epitope for the Wright^b antibody used is thought to be on the Band 3 portion of the Wright^b antigen, and as the GYPA KO do express Band 3, this binding is to be expected. However, as discussed in the introduction, GYPA is required for the proper formation of the Wright antigen (Bruce et al., 1995), therefore it is unlikely that the Wright^b detected on the GYPA KO cells is of normal structure or function (Bruce et al., 1995). On IFA, Wright^b binding was noticeably reduced compared to wildtype with an altered pattern (Figure 5-17). This is in keeping with the evidence that that GYPA has a role in trafficking Band 3 to the cell surface and promoting clustering of Band 3 molecules (Kalli and Reithmeier, 2018). In my experiments Band 3 expression on the GYPA KO cells appeared slightly reduced to the wild type cells on flow cytometry, though the differences were not obvious on IFA (Figure 5-16 and Figure 5-17).

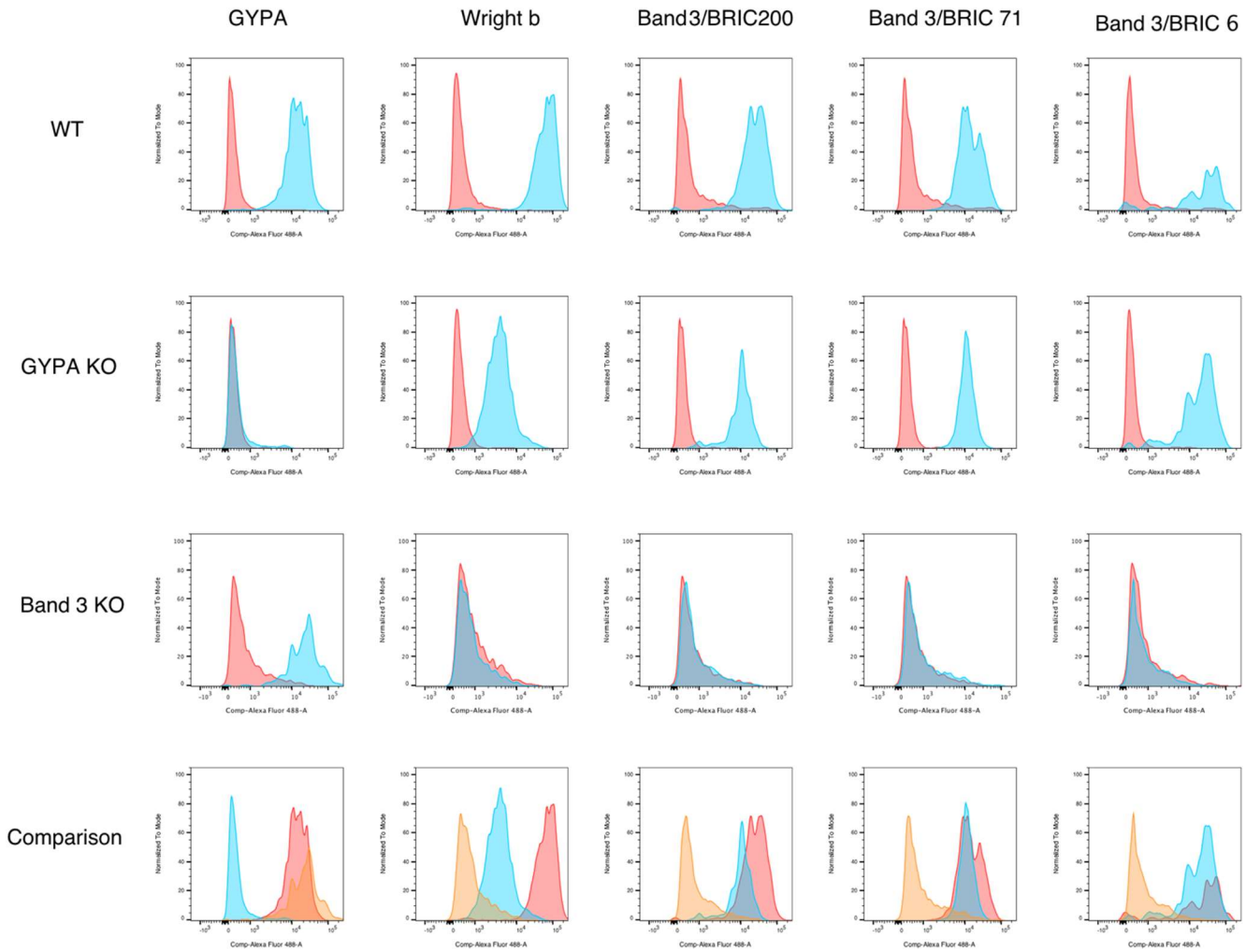


Figure 5-16 Band 3, Wight[b] and GYPA expression on KO EJ cells. Three different Band 3 antibodies were used (BRIC 200, BRIC 71 and BRIC 6). RED- isotope control, BLUE- receptor. For comparison plots RED- WT, BLUE- GYPA KO, ORANGE- Band 3 KO. All plots have been normalized to mode.

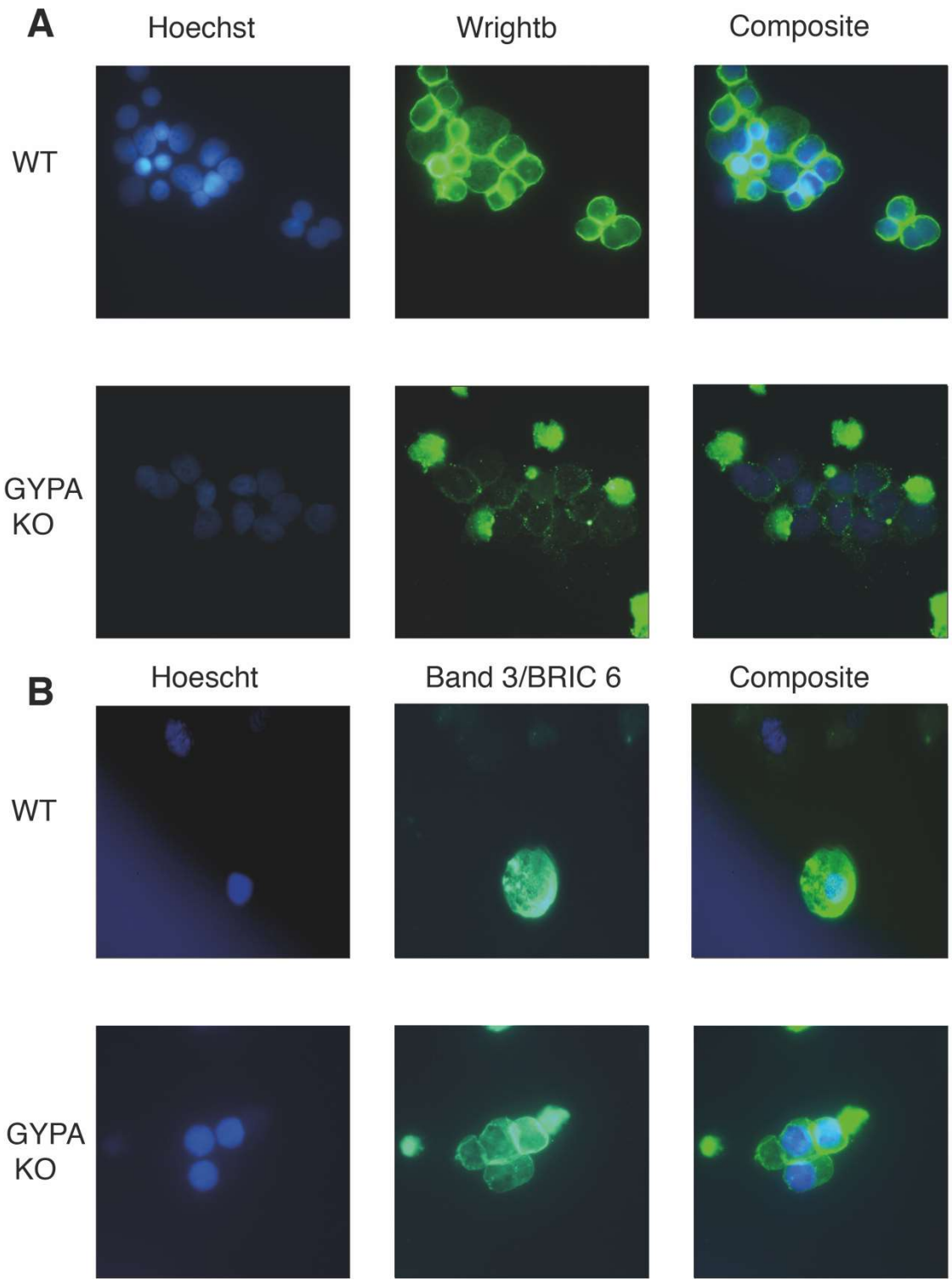


Figure 5-17 IFA of Wright^{b} (A) and Band 3 (B) expression on WT and GYPA KO EJ cells. BLUE- Hoechst nuclear stain, GREEN- Alexa488. Images were taken using the x100 objective on a Leica DM LB2 florescence microscope with a Yenway microscope camera. The same exposure was used for Hoechst and Alexa488 images for each cell type. Composite images were made using ImageJ software. Of note, the very brightly fluorescing cells shown in the GYPA KO Wright^{b} panel are dead cells.

5.5.5 Rosetting of CR1, GYPA and Band 3 KO EJ cells

5.5.5.1 Rosetting of CR1 KO EJ cells

No significant differences were seen in rosette frequency between CR1 knockout EJ cells, wildtype EJ cells and unmatched peripheral erythrocytes, though there was a trend towards decreased rosetting with R29 (Figure 5-18). This is perhaps not surprising given the already very low level of CR1 expression on wildtype EJ cells.

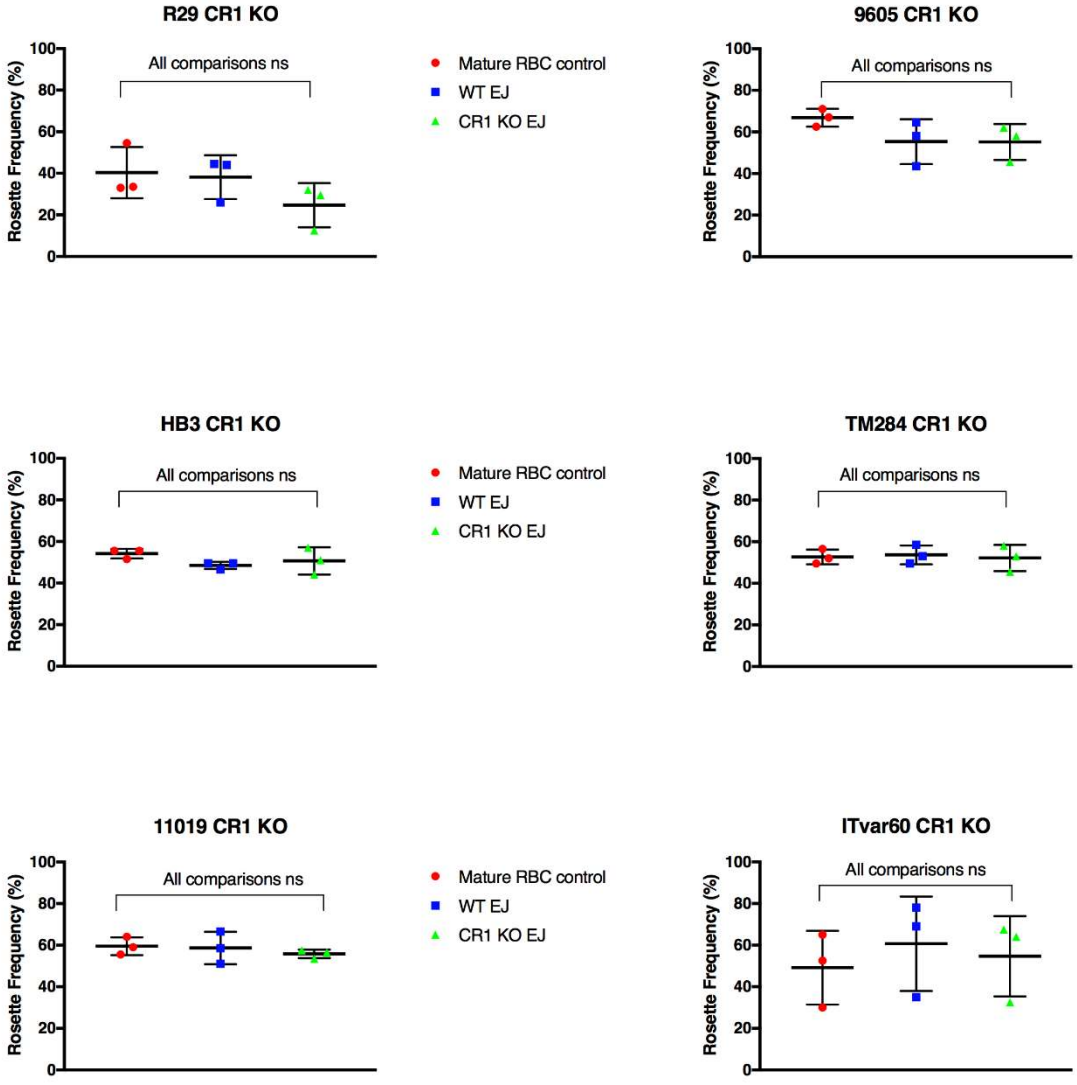


Figure 5-18 Rosetting of CR1 KO EJ cells compared to wildtype EJ cells and unmatched peripheral erythrocytes. Each data point represents the results of one independent, blinded experiment (the mean of two technical replicates as described in the methods section 2.6). p-value <0.05 was considered significant. Error bars show the mean and standard deviation of the three results.

5.5.5.2 Rosetting of GYPA and Band 3 KO EJ cells

The rosetting of both GYPA (Figure 5-19) and Band 3 KO EJ cells (Figure 5-20) was reduced for all strains tested, though for the Band 3 KO with TM284 this did not reach significance, and 9605 was not tested with Band 3 KO cells. Of note, unlike the cRBC, no difference was seen in the rosetting ability of wildtype EJ cells and peripheral erythrocytes. Also of interest, no significant differences were seen in the (reduced) rosetting frequencies between GYPA and Band 3 KO cells (Figure 5-21), indicating that neither the presence of Band 3 in the GYPA KO, nor GYPA in the Band 3 KO, are capable of rescuing the rosetting phenotype. This suggests that it is the Wright^{b} antigen, absent in both KO cells, that is key for rosetting. However, it is important to note that Band 3 structure and function is not entirely normal in the absent of GYPA and the absence of Band 3 will have a significant impact on the structure and function of the erythrocyte(Bruce et al., 1994, Bruce et al., 2004); this will be further discussed in the following section.

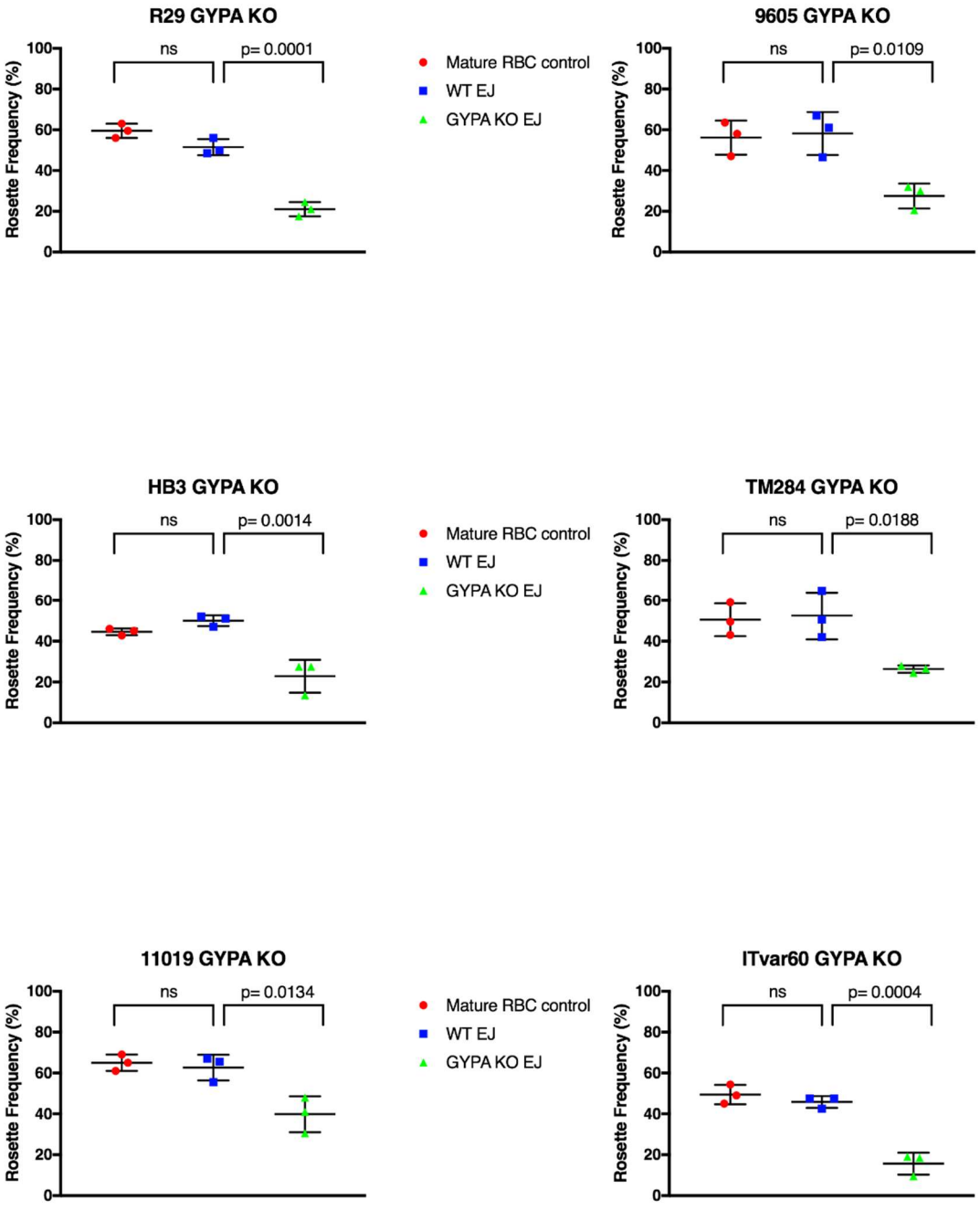


Figure 5-19 Rosette frequency is significantly reduced with GYPA KO EJ cells. Each data point represents the results of one of three independent, blinded experiments (the mean of two technical replicates as described in the methods section 2.6). p-value <0.05 was considered significant and were calculated using an ordinary one-way ANOVA with Tukey's multiple comparisons test. Error bars show the mean and standard deviation of the three results

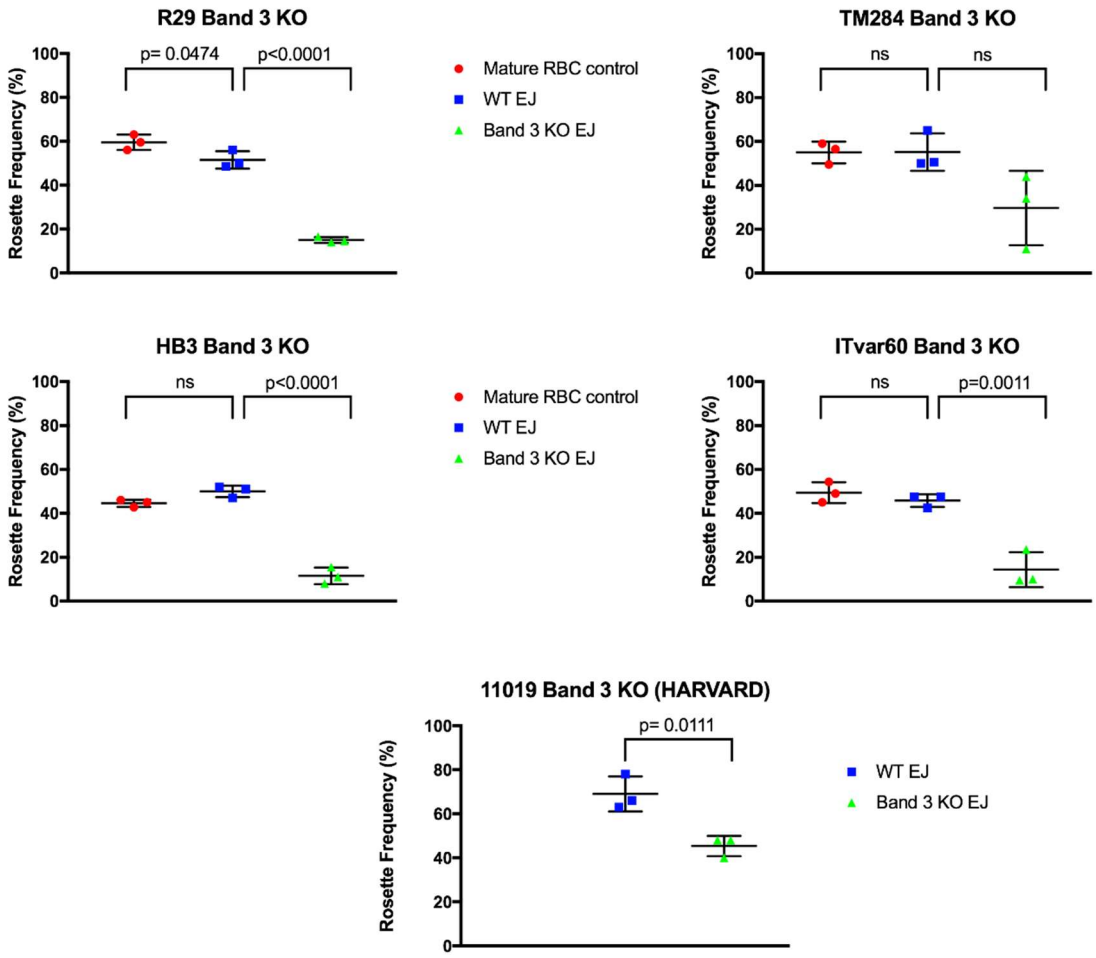


Figure 5-20 Rosette frequency is reduced with Band 3 KO EJ cells. Each data point represents the results of one of three independent, blinded experiments (the mean of two technical replicates as described in the methods section 2.6). The experiments for 11019 were carried out in Boston and did not include mature peripheral erythrocytes. p-value <0.05 was considered significant and were calculated using an ordinary one-way ANOVA with Tukey's multiple comparisons test, with the exception of the Harvard data for 11019 which was analysed using an unpaired t-test. Error bars show the mean and standard deviation of the three results.

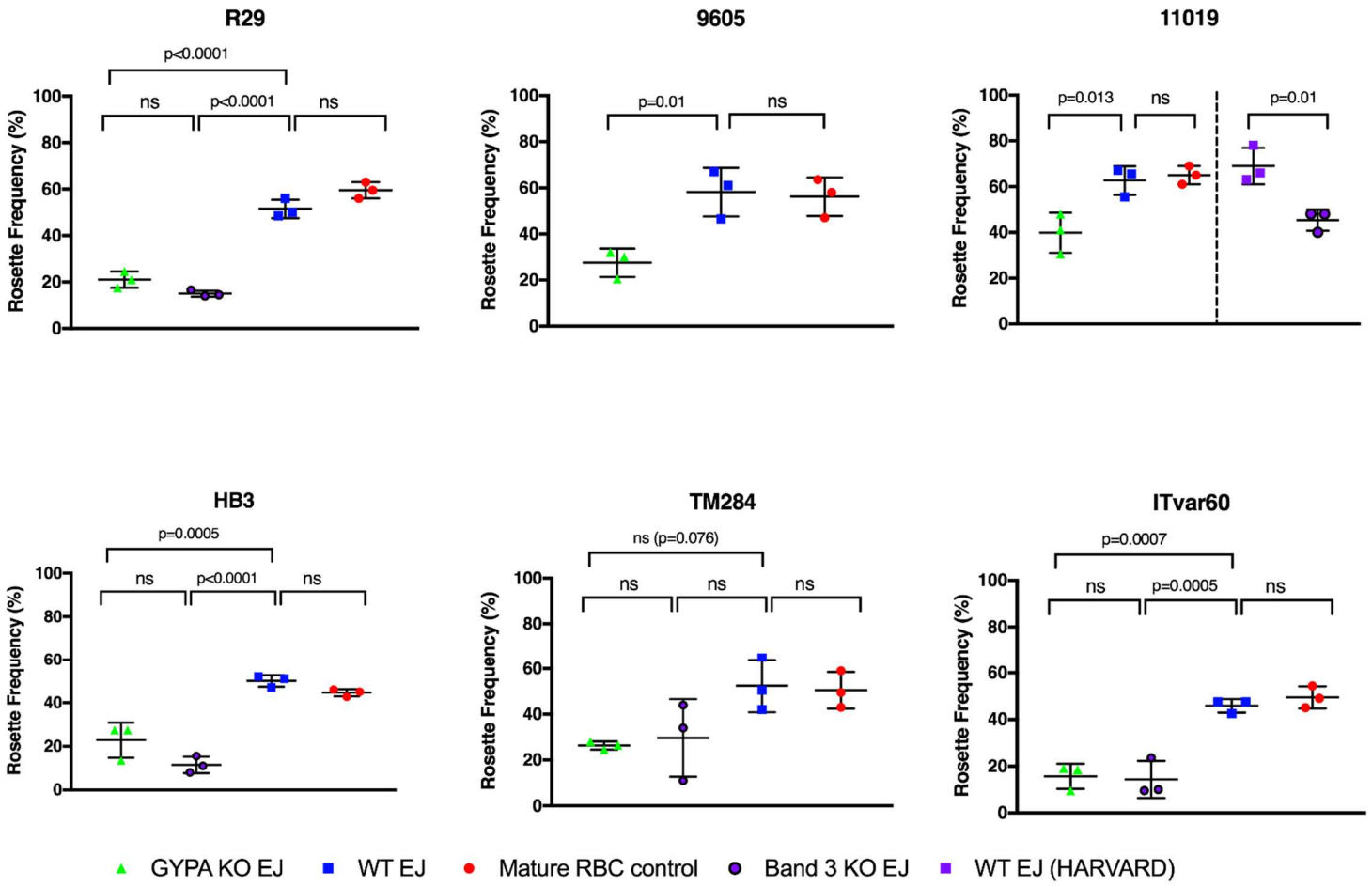


Figure 5-21 Combined GYPA and Band 3 KO EJ cells. Data shown are the same as those in Figure 5-19 and Figure 5-20, the experiments were carried out in parallel (except for 11019 as described), using the same WT EJ cells and peripheral erythrocytes therefore are directly comparable. Each data point represents the results of one of three independent, blinded experiments as before. Data were collectively re-analysed using an ordinary one-way ANOVA with Tukey's multiple comparisons test, with the exception of the Harvard data for 11019 which was analysed using an unpaired t-test. Error bars show the mean and standard deviation of the three results

5.6 Discussion

The data presented here support the hypothesis that the Wright^b antigen is a key, strain-transcending host rosetting receptor for *P. falciparum*, however there are important limitations regarding the use of GYPA/BAND 3 KO cells which are further discussed below. In keeping with the rosette disruption demonstrated by anti-Wright^b antibodies, mature EJ cells lacking Wright^b had significantly reduced rosetting frequencies compared to wildtype EJ cells, suggesting that Wright^b could be a valuable anti-rosetting therapeutic target. In addition, the EJ cells have proved to be an extremely useful tool with great future potential.

5.6.1 Further investigation into Band 3/Wright^b as a rosetting receptor

While these data are encouraging, there are many questions that remain to be answered. In particular, are the effects of the Wright^b antibodies also seen in parasites grown in blood group A, or do the stronger and larger rosettes seen in some strains when cultured in their preferred blood group ameliorate the effect? A useful therapy would be required to work in all blood groups, particularly as the non-O groups are particularly associated with severe malaria (Rowe et al., 2007, Tekeste and Petros, 2010, Rout et al., 2012, Network, 2014). This phenomenon may also be relevant for the differing results seen in the existing literature in which Rowe *et al.* (Rowe et al., 1997) tested M^{k}M^K cells for rosetting as part of a rare erythrocyte panel and found that these cells were capable of forming rosettes, in contrast to that of my GYPA knockouts. The blood group of the tested cells is not certain (A. Rowe, personal communication) and it is possible that if they were of a non-O blood group (unlike my EJ cells), the blood group antigens could have compensatory effect which would allow rosetting even in the absence of Wright^b.

As yet, I have not investigated the parasite side of the rosetting equation and it is not known which parasite-derived ligands bind to Band 3/Wright^{b}. Band 3, along with GYPA and Wright^{b}, can be expressed in *Xenopus* oocytes and K562 cells (Groves and Tanner, 1992, Groves et al., 1993, Beckmann et al., 1998, Young et al., 2000) Therefore these cells could be used in binding assays with *P. falciparum* infected erythrocytes, to assess whether Band 3/Wright^{b} is sufficient for rosetting. The specific parasite ligands could be investigated further by mildly trypsinising the infected erythrocytes to remove PfEMP1, and monitoring the effect on binding (Kyes et al., 1999). If infected erythrocytes are still able to bind, this suggests trypsin-resistant parasite ligands, such as the RIFINs are the key binding molecule. If, however, PfEMP1 was found to be the key ligand, recombinant PfEMP1 domains could be used to determine the precise binding domains, bearing in mind the caveats to using recombinant PfEMP1 discussed throughout this thesis.

Given the overall aim of rosetting research is to reduce the mortality and morbidity of severe malaria, thought must also be given to translating these findings into a potential anti-rosetting therapy, and the prospect of this will be further discussed in Chapter 6.

5.6.2 Limitations of the EJ rosetting model

Despite these promising findings, it must be acknowledged that the EJ cells are not a perfect proxy for mature peripheral erythrocytes. EJ cells are larger and, in the vast majority of cases, nucleated which has consequences for deformability (Guzniczak et al., 2017). Though Trakarnsanga *et al.* report that the BEL-A cells had similar deformability to peripheral erythrocytes, they only tested filtered, enucleated BEL-A cells (Trakarnsanga et al., 2017), while most of the cells I used in my rosetting assays still contained a nucleus. The difference in rosette morphology can be seen in Figure 5-22 which shows an EJ rosette with a 11019 parasite-infected erythrocyte, compared to a normal rosette. EJ cells do not appear to adopt the ‘plastered on’ appearance of

normal erythrocytes onto the infected cell and instead adhere over a smaller cell surface area.

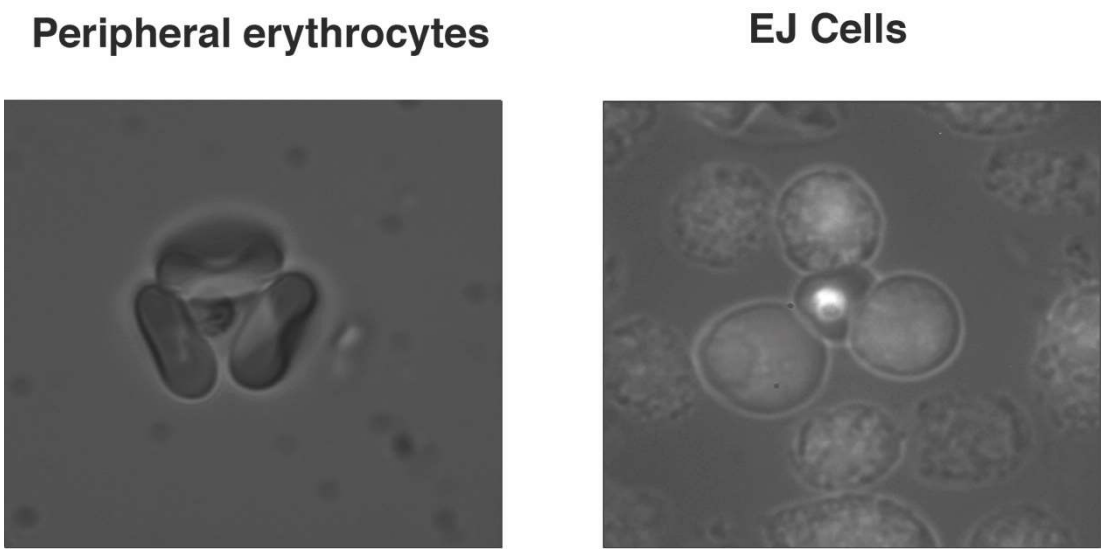


Figure 5-22 Comparison of rosette morphology for mature peripheral erythrocytes and EJ cells. In both cases, there is a central, infected erythrocyte, surrounded by three, uninfected cells. Images were taken using a Leica DM LB2 florescence microscope and have been cropped from a larger image taken using the x20 objective. Images have been changed from colour to monochrome using Image J software.

The other major caveat which applies to the EJ cells specifically is that even the wildtype EJ cells do not come from a 'normal' donor, as the EJs were immortalised from the PBMC of a patient with haemochromatosis. While there is no obvious reason to suspect that this would affect the results, particularly as the knockout cells are compared to wildtype EJ cells from the same source, it would be useful to confirm these findings with the BEL-A cells which derive from a healthy original donor, when they become available.

While the knockout EJ cells have been profiled for the receptors of particular interest in terms of rosetting, I cannot exclude the possibility of off-target effects arising from the CRISPR/Cas9 gene editing. In addition, pure Wright^{b} knockout cells were not available, and the conclusion that Wright^{b} is the key molecule has been inferred from cells which were abnormal in other respects (e.g. lacking GYPA and Band 3). However, the similarities in rosetting

frequency between the GYPA and Band 3 knockouts do suggest that the common factor, i.e. lack of Wright^{b} was crucial.

Aside from the potential off-target effects of gene editing, the removal of either Band 3 or GYPA has significant structural consequences for the erythrocyte. The loss of Band 3 leads to disruption of the Band 3/Ankyrin macrocomplex and loss of additional proteins including protein 4.2, GYPA, Rh antigen, CD47, LW and GYPB (Kalli and Reithmeier, 2018). Band 3 null cells are spherocytotic and fragile (Toye et al., 2008), which likely accounts for the marked cell loss noted during the later stages of culturing. Similarly, GYPA null cells have altered Band 3, protein 4.1 and p55, with reduced cell volume (Bruce et al., 1994, Bruce et al., 2004). All of these features could, in theory, affect the rosetting ability of the KO cells, independent of their Wright^{b} status. Rather than wildtype EJ cells, more appropriate controls would be those which also had a disrupted macrocomplex but still expressed Wright^{b}, for example a RhAG KO as the Band 3 KO control or Protein 4.1 for the GYPA (Professor L Bruce, personal communication).

I also fully acknowledge the lack of sequencing data for the knockout cells which could confirm the genetic, rather than just phenotypic, knockout of the cells. However, arguably, this is less important that the actual receptor profile which I have assessed through antibody binding and flow cytometry. As the GYPC knockout shows, an apparent genetic knockout by sequencing does not necessarily mean that no GYPC is expressed on the cell surface.

There are other, technical, issues with the EJ cells, particularly the massive cell loss at the late stages of differentiation, however this may be an issue with many of the immortalised cell lines (see Trakarnsanga *et al.* 2017 supplemental information figure 1). Culturing is also expensive, particularly when maintaining large cell numbers.

5.6.3 Generating multiple erythroid knockouts and screening for novel receptors

Despite these limitations, the EJ cells, and other immortalised erythroid lines, offer a great range of opportunities for the malaria field as a whole, including research into host invasion and sequestration receptors. Of particular interest in the context of rosetting, the generation of multiple knockouts could help dissect the relative contribution of various rosetting candidates to better determine which are essential and which have a more accessory role. Such knockouts have been designed by the Bristol BEL-A team, though the focus of these cells was to create universal erythrocytes for blood transfusion (Hawksworth et al., 2018). Various combinations of knockouts of the known rosetting receptors could be tested to determine which receptors were necessary and/or sufficient. If a cell that lacked CR1, AB antigens, GYPA (and therefore Wright^{b}) and GYPC was capable of rosetting, this would suggest the existence of further, as yet undetermined, host rosetting receptors. The EJ cells are also potentially a tool for screening for these unknown rosetting receptors, in a similar manner to the invasion receptor screen undertaken by Egan and colleagues (Egan et al., 2015) and this will be discussed further in Chapter 6.

To conclude, the data generated using the knockout EJ erythroid cells corroborates the results of the Fab experiments and both findings support the hypothesis that the Wright^{b} antigen is an important, strain-transcending host rosetting receptor. There is more work to be done in elucidating the parasite side of the rosetting equation and confirming that the same results apply in different ABO blood groups. However, this discovery represents an important new piece of the rosetting puzzle which has potential to lead to the development of a specific anti-rosetting therapy for use in severe malaria.

6. CHAPTER VI: CONCLUSIONS

P. falciparum malaria is a pernicious disease, affecting millions of children in some of the poorest countries of the world. Around 10% of children with malaria in Africa will develop severe disease, which has an unacceptably high morbidity and mortality. At present, we have no effective adjuvant treatments for severe disease and with the advent of increasing anti-malarial drug resistance, the scale of the problem is likely to worsen.

The goal of my research is to better understand the pathological mechanisms of rosetting in severe malaria, with a view to informing the development of novel adjuvant therapies to reduce the mortality and morbidity of this deadly condition. I approached this challenging problem by first re-evaluating current assumptions regarding the role of the glycosaminoglycans, heparan sulfate and chondroitin sulfate, in rosetting. Having concluded that that neither of these molecules represents a specific host erythrocyte rosetting receptor, I then sought to overcome the limitations of existing experimental methodologies by establishing a new approach using immortalised knockout erythrocytes. During the development of these techniques, I identified a new potential rosetting receptor, Wright^{b}, and was able to confirm the importance of this molecule for rosetting across all strains tested, using two different experimental approaches; rosette disruption with anti-Wright^{b} antibodies and the use of CRISPR-Cas9 knockout erythrocytes.

6.1 Translating my results to the clinic

These results are tremendously exciting as they suggest that a therapy directed against Wright^{b} could possibly reduce rosetting in severe malaria and potentially alleviate some of the microvascular obstruction thought to contribute to the disease pathology. However, there are a number of questions to be addressed before such a therapy reaches the clinical research stage, and further work is required to better understand the mechanisms of this host-parasite interaction. These questions are summarised in Table 6-1.

Table 6-1 Questions to be addressed when considering the translation of my findings into an effective clinical therapy

Clinical questions	Mechanistic questions
What are the potential pathological consequences of releasing rosettes?	Can we demonstrate direct binding between Wright^{b} and infected erythrocytes?
How would blocking Wright^{b} affect the erythrocytes?	To which parasite ligand does Wright^{b} bind?
At what stage of disease would the treatment be most effective?	Is the same level of rosette disruption by anti-Wright^{b} seen in different blood groups?
Would rosette disruption be sufficient for a therapeutic effect?	Does blocking Wright^{b} have any effect on sequestration?
Cost, availability, duration of treatment etc.	Do rPfEMP1 co-localise with Wright^{b}?

6.2 Clinical questions

If one accepts the theory that rosetting is a causative factor in the pathology of severe malaria, possibly by contributing to microvascular obstruction, it seems logical that disrupting these rosettes would be beneficial to the patient. However, this assumption must be carefully considered, as it is also plausible that the sudden release of erythrocytes infected with mature parasites into the peripheral circulation may be detrimental. In particular, the spleen could become overwhelmed with infected erythrocytes leading to a splenic sequestration crisis as seen in sickle cell disease. Though this is perhaps less likely for a therapy which would specifically release rosettes, rather than causing generalised desequestration of infected cells. Encouragingly, Leitgeb *et al.* saw no such issues in their clinical trial of Sevuparin, despite demonstrating transient desequestration, though splenic size or ultrasound assessment of the spleen was not done (Saiwaew et al.,

2017). Of note, this trial was in adults with uncomplicated malaria and relatively low parasitaemia (10,000-100,000/μl). Dondorp *et al.* (Dondorp et al., 2007) reported similar findings in patients with uncomplicated malaria using the anti-helminth drug, Levamisole, an alkaline phosphatase inhibitor which inhibits cytoadhesion. Patients treated with Levamisole had higher levels of erythrocytes infected with mature forms of the parasite in the periphery, again with no safety concerns. However when Levamisole was trialled in patients with severe malaria, no desequestration was seen (Maude et al., 2014), therefore the consequences of releasing rosettes in children with severe malaria remain unclear. Even if no adverse effects were seen, it is also uncertain whether simply disrupting rosettes would be sufficient to reverse the pathology of severe disease. However, field studies have demonstrated that a relatively small decrease in rosette frequency does impact on disease severity (Rowe et al., 1995, Rowe et al., 2007, Doumbo et al., 2009, Rout et al., 2012), and my results show that anti-Wright^{b} Fab were highly effective at disrupting the majority of rosettes.

Perhaps more concerning is the potential effect of anti-Wright^{b} antibodies/Fab on the uninfected host erythrocytes. As whole anti-Wright antibodies cause significant agglutination and extravascular haemolysis (Squires et al., 2012), it would be necessary to use antibody fragments, however even Fab may have clinically important clinical consequences. Early work by Pasvol *et al.* (Pasvol et al., 1989) showed that the addition of anti-Wright^{b} Fab at 50μg/ml (half the concentration used in this thesis) reduced the deformability of erythrocytes, though the physiological consequences of this are unknown and more work is required to explore this further. Interestingly, the anti-Wright^{b} Fab also significantly reduced parasite invasion, and, given that erythrocytes lacking Wright^{b} had previously been found to be fully susceptible to invasion (Hermentin et al., 1985), the mechanism was felt to relate to the decrease in cell deformability (Pasvol et al., 1989). It is therefore possible that an anti-Wright^{b} antibody, anti-rosetting therapy could have the additional benefit of reducing invasion. However, it is

also important to consider whether my rosette disruption results could be due to this reduced deformability mechanism. The additional evidence provided by the knockout EJ cells, which did not rely on the Wright^{b} antibody, suggest that this is not the case, however as discussed in section 5.6.2 there are important caveats to my finding as the wildtype EJ cells were not the most appropriate experimental controls. Therefore, there are alternative explanations for both the Fab and EJ KO results and it is not possible to definitively confirm that Wright^{b} is a specific host rosetting receptor. Nevertheless, Wright^{b} Fab do disrupt rosettes, just as Heparin does (albeit Heparin acts through a non-specific mechanism), and it these Fab are found to be safe, they could still represent and important and useful anti-rosetting therapy. .

On that note, before testing any potential anti-rosetting therapy in humans, a phase of animal testing must be completed. The literature regarding the use animal models of *P. falciparum* is large and much debated (Craig et al., 2012), and as such will not be discussed in further detail here. However, advances in the development of humanised mouse models (Rahmig et al., 2016, Foquet et al., 2018, Minkah et al., 2018) may address some of these issues and offer a useful method of testing an anti-Wright^{b} therapy *in vivo*.

6.3 Mechanistic questions and future work

I addressed the question of the role of Wright^{b} in rosetting using two different approaches; antibody mediated rosette disruption and the use of knockout cells. This allowed me to assess the therapeutic potential of using anti-Wright^{b} to disrupt pre-formed rosettes and establish that Wright^{b} is a key host erythrocyte rosetting receptor. However, I have yet to demonstrate that Wright^{b} is sufficient for rosetting or determine to which parasite-derived ligand Wright^{b} binds. This latter question is particularly interesting as a recent study (Almukadi et al., 2019) has suggested that *Plasmodium falciparum* glutamic acid-rich protein (PfGARP) binds to Band 3 (though a different

epitope to Wright^{b}) which the authors suggest may be related to rosetting. In section 5.6.1 I have outlined plans to express Wright^{b} in alternative cell types and assess the binding of PfEMP1 and other parasite ligands. In addition to these experiments, recombinant PfGARP domains could also be used. I have also discussed the need to test the effect of anti-Wright^{b} in parasites cultured in blood group A, and encouragingly, preliminary experiments by Molly Carlier and Professor Rowe appear to show the same effect regardless of blood group (personal communication).

Another potentially interesting avenue to explore could be the role of Band 3/Wright^{b} in sequestration. Band 3/Wright^{b} is only expressed on erythrocytes and in the renal tubules, not on endothelial cells, therefore one would not expect to see a desequestration effect with anti-Wright^{b}. However, others have suggested that modified Band 3 may act as an adhesion molecule, not on the host erythrocytes, but on the infected cells (Winograd and Sherman, 1989, Eda et al., 1999, Winograd et al., 2005). Sherman, Winograd and colleagues (Sherman et al., 1995, Sherman et al., 2003) propose that erythrocyte infection with *P. falciparum* induces conformational changes in Band 3 (Eda et al., 1999, Winograd and Sherman, 2004) which exposes an epitope, Pfalhesin, capable of binding to the endothelial receptors Thrombospondin (TSP) (Lucas and Sherman, 1998, Eda et al., 1999) and CD36 (Winograd et al., 2004). Binding to TSP and CD36 in cytoadhesion assays on plastic dishes is common in clinical isolates, but not particularly associated with severe disease (Heddini et al., 2001). This adhesion can be disrupted *in vitro* with antibodies or synthetic peptides of the Band 3 sequence, HPLQKTY (found on the third extracellular loop) (Lucas and Sherman, 1998, Eda et al., 1999). In addition, the infusion of synthetic peptides based on residues 546-553 (GHPLQKTY) and 553-546 (YTKQLPHG) causes desequestration in infected *Aotus* and *Saimiri* monkeys (Crandall et al., 1993). These sites are some distance from the Wright^{b} antigen, however an assessment of the ability of anti-Wright^{b} to interrupt binding to endothelial cells may be an interesting experiment to conduct.

6.4 A rosetting screen

In addition to Wright[b], there may be other important rosetting receptors which have not yet been discovered. The development of immortalised erythroid cell lines, such as the EJ cells, will help remove many of the technical barriers which have hampered progress thus far, and a rosetting screen, similar to that of Egan *et al.* (Egan et al., 2015) could be a very useful experiment. In their quest to identify novel invasion receptors, Egan and colleagues transfected CD34+ cells with a pooled shRNA lentiviral library before selecting and differentiating the resulting knockdown cells. The transfected erythrocytes were then infected with GFP-expressing, 3D7 *P. falciparum* parasites or mock infected, and the shRNA between the infected erythrocytes (selected by GFP positivity) and mock infected cells were compared (Egan et al., 2015). In this way, CD55 was found to be an essential *P. falciparum* invasion factor. Such a screen for rosetting receptors would be more challenging, as isolating rosettes is not as simple as sorting by GFP expression. Flow cytometry has been used to detect multiplets as a surrogate measure of rosettes (Ch'ng et al., 2016), however the reliability of these methods and the relationship between multiplets and rosetting frequency has not yet been fully established. Alternatively, cells could be sorted by those which bind fluorescently tagged recombinant rosetting PfEMP1 domains. This would have to include the caveat, illustrated throughout this thesis, that PfEMP1 binding may not always be an accurate representation of binding to infected erythrocytes. Nevertheless, there is much potential in the idea of a rosetting screen of this kind, and we are in discussion with the Duraisingh laboratory on how to proceed with such an experiment using the EJ cells.

6.5 Limitations

Many of the limitations of this work have been discussed in the relevant chapters. However, one overriding limitation to my findings is that all my experiments have been carried out using laboratory adapted parasite lines, some of which have been in culture for decades and may not have originated from Africa or from patients with severe malaria. The lines 11019 and 9605 are more recently adapted and do come from African children with severe disease, but a key experiment which I have yet to carry out is testing fresh clinical isolates from the field. It is these such isolates which are clinically relevant and representative of the parasites causing severe disease in 'real-life'. My original plans to spend time studying such isolates in Uganda have been modified, mainly due to family reasons, nevertheless, I am conscious of the importance of establishing that my results are not simply an artefact of using laboratory parasites and are just as applicable to those in the field.

I am also conscious that, while the EJ and cRBC have been a very useful tool, they are not a perfect representation of mature peripheral erythrocytes. In particular, the vast majority of the EJ cells remain nucleated and therefore can be expected to have altered physical properties in terms of size and deformability. Additionally, as has been demonstrated by myself and others (Wilson et al., 2016, Dankwa et al., 2017), the cell surface receptor complement of these cultured erythroid cells is not identical to that of mature erythrocytes. Therefore, the results of experiments using these cells must be interpreted with these differences in mind, and corroborating evidence using mature erythrocytes should also be sought.

Finally, there remains another conflicting result from the literature which I cannot fully explain. In section 4.2.2.1 I discussed a study by Rowe *et al.* (Rowe et al., 1997) in which multiple, rare red cell variants were tested for rosetting ability. One such variant, M^kM^k, lacks GYPA and GYPB, therefore, by extension presumably lacks Wright^b, however these cells were found to

rosette normally. Only one donor was used, and the blood group of the donor was not entirely clear (A. Rowe, personal communication), therefore it is possible (if the donor was non-O) that rosetting via the blood group A antigens is sufficient for rosetting, even in the absence of Wright^{b}. As an alternative to my knockout EJ cells, a useful experiment would be to retest these cells, along with other rare variants, in particular those of the Dantu and En(a-), MiV and Wright^{a} phenotypes (Table 5-2). I have also not explored the potential involvement of the very rare Wright^{a} phenotype in rosetting. One might expect that if Wright^{b} is key for pathological rosetting then negative selection for this antigen would occur and Wright^{a} would be more common, particularly in African populations. Wright^{a} is extremely rare in Caucasian populations (Bruce et al., 1995), but comprehensive data on its prevalence in Africa is lacking, though the polymorphism has not been highlighted as a specific protective factor in large Genome Wide Association Studies (Leffler et al., 2017, Band et al., 2015). A useful further experiment would be to test and compare the rosetting abilities of various Wright antigen phenotypes including Wright$^{(a+b+)}$ and particularly Wright $^{(a+b-)}$.

6.6 Final thoughts

Severe malaria is a complex phenomenon. Even after over a century of research the questions of why some people experience severe disease, who gets it and how the pathological processes develop remain, at least partly, unanswered. Rosetting has been associated with all types of severe malaria and represents a potential therapeutic target. The parasite ligands involved in rosetting are highly variable and as yet, the development of a strain-transcending vaccine remains challenging (Ghumra et al., 2011, Ghumra et al., 2012). I have focused on the other side of the rosetting equation, in a quest to discover the host receptors essential for rosetting in severe *P. falciparum* malaria. Through re-evaluating the existing literature, I have challenged the dogma that heparan sulfate is an important rosetting receptor but have also identified a novel rosetting receptor which appears to be important across multiple strains. The next stage of my research is to delve deeper into the mechanisms of Wright^{b} mediated rosetting and explore the potential for an anti-Wright^{b} therapy. Ultimately, I want to translate my findings into an effective treatment which can be used to reduce the mortality and morbidity from childhood malaria in the near future.

7 APPENDICES

7.1 Flow cytometry gating strategies

7.1.1 Chapter 3

7.1.1.1 Gating strategy for Figure 3-16

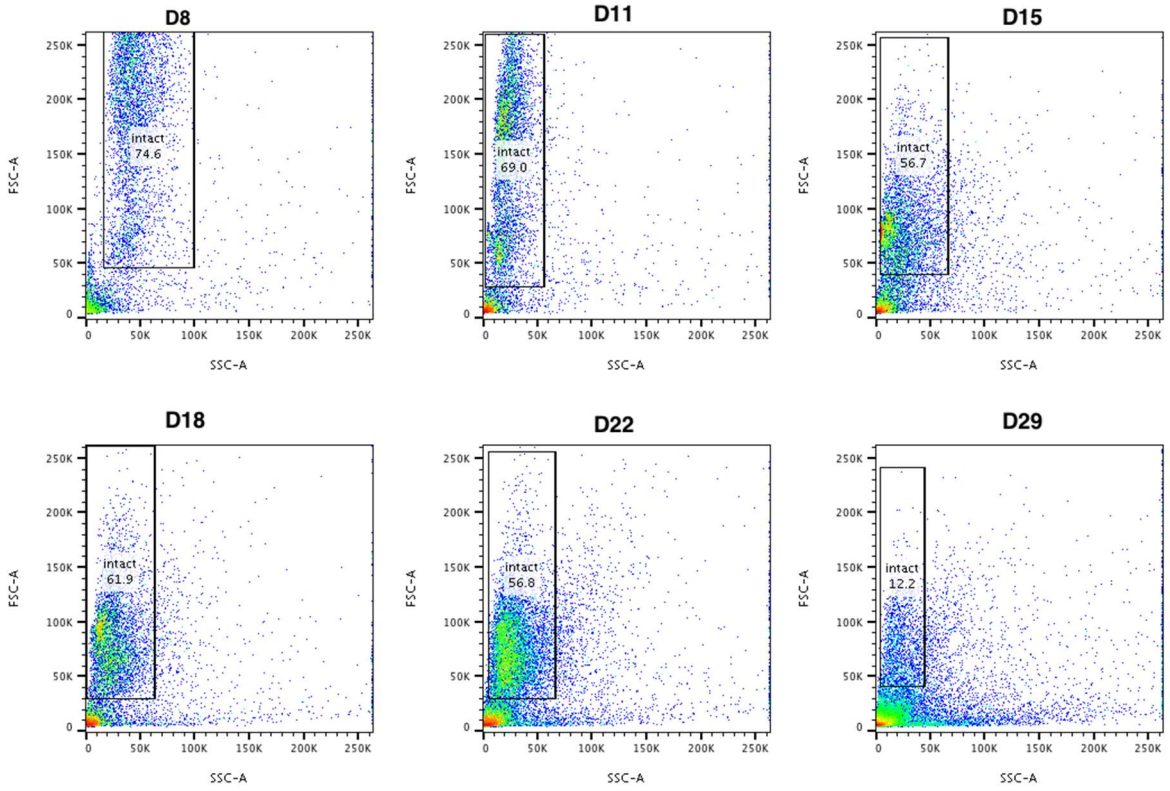


Figure 7-1 Gating strategy for Figure 3-16 The IgG2bk isotype control labelled cells are showed and were gated by FSC and SCC to exclude debris. No live/dead staining was used for this experiment.

7.1.1.2 Gating strategy for Figure 3-17

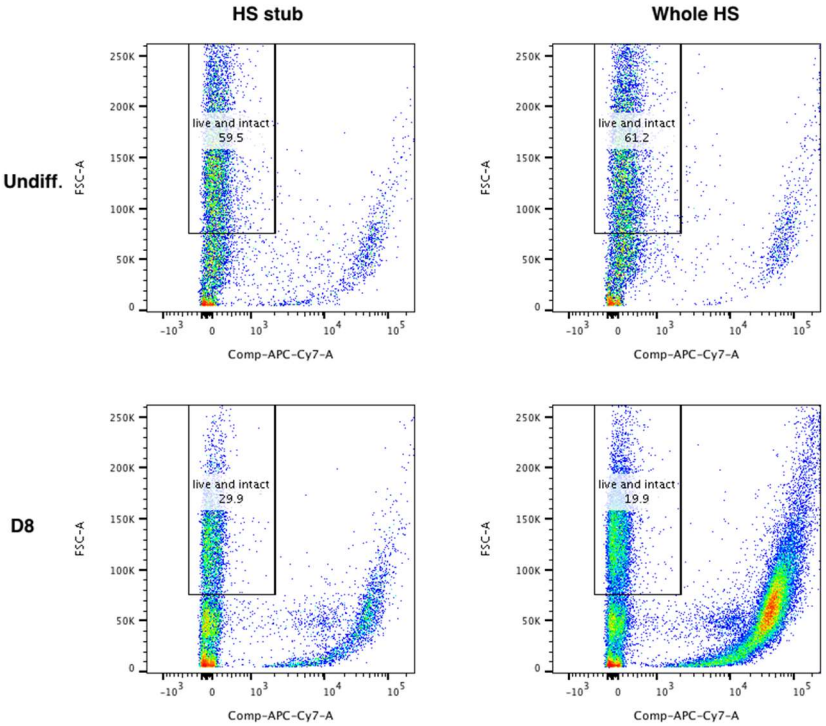


Figure 7-2 EJ cells gating strategy for Whole HS and HS stubs when undifferentiated and at D8. Note: the "Comp-APC-Cy7-A" is the Live/Dead strain, with high levels indicating dead cells.

7.1.2 Chapter 4

7.1.2.1 Gating strategy for Figure 4-12

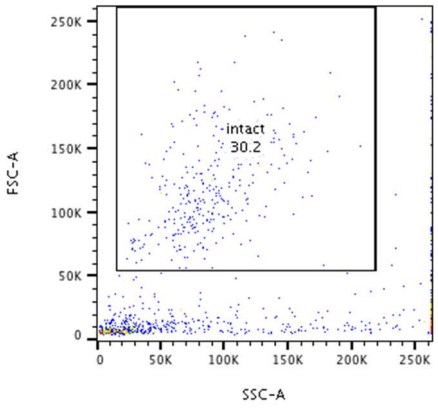
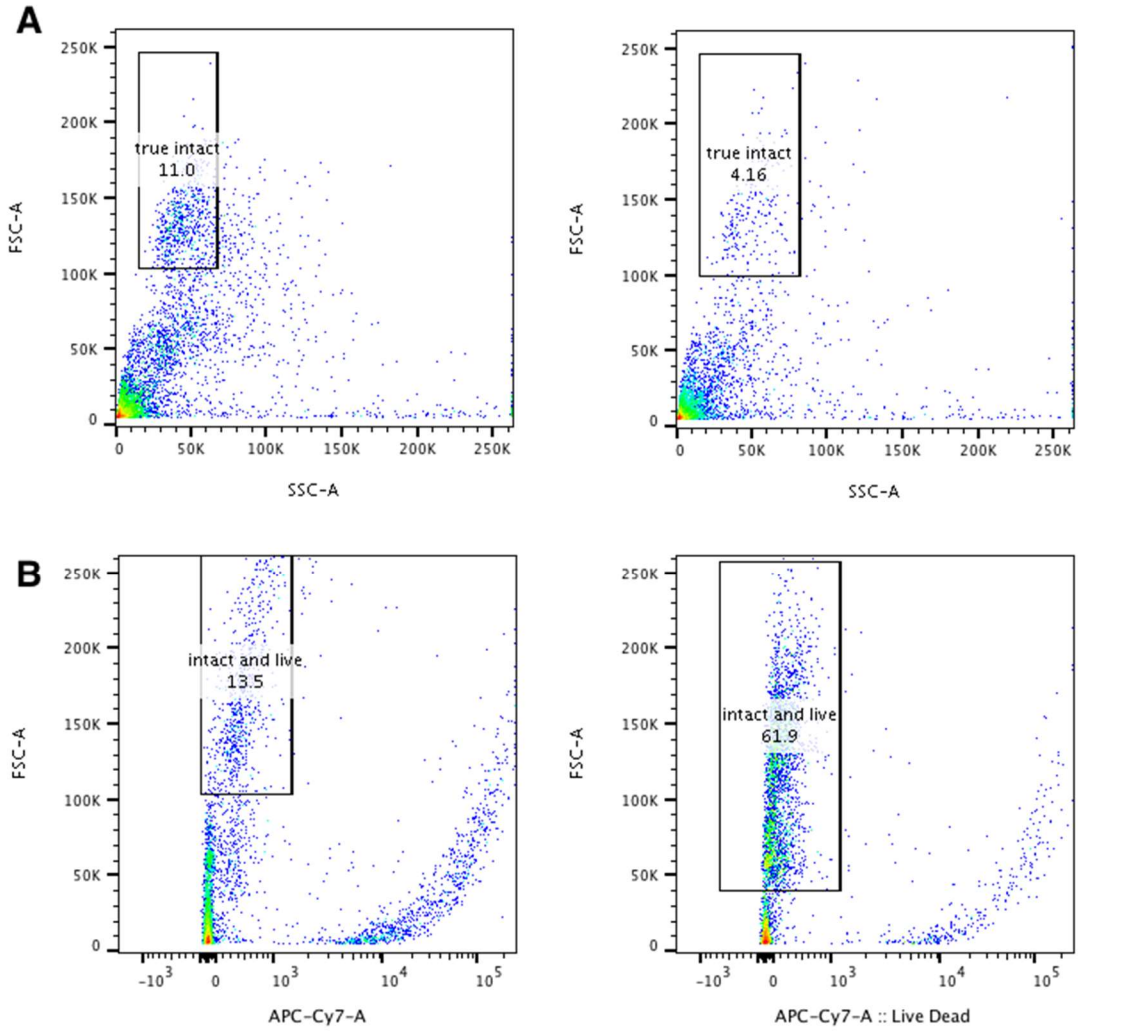


Figure 7-3 Gating strategy for day 4 CD34+ cRBC. Isotype control labelled cells are shown and gate was copied over to CD34 labelled cells

7.1.2.2 Gating strategy for Figure 4-23



Plots show control transduced cells, gates were copied over to knockdown cells, therefore control and knockdown comparisons in chapter 4 are using indentical gates.

Figure 7-4 Flow cytometry gates for flow plots shown in Figure 4-23, lentiviral transduction results. Plots shown are for control cells and are gated for intact cells by FSC and SSC (A) or FSC and live/dead stain (B)

7.2 Additional experiments

7.2.1 Chapter 4 Comparison of different multiplicity of infections for GYPC transduction

Cells were transduced as described in section 4.4.7, however three different multiplicities of infection (MOI) were used for both control and knockdown cells. The control lentiviral particles incorporate GFP expression into the cells while the GYPC shRNA do not. For MOI 0.5 (transducing units [TU] $5x10^4$), 10µl of virus was added, for MOI 1 (TU $1x10^5$), 20µl of virus was used and for MOI 5 (TU $5x10^5$), 100µl of virus was used. The corresponding volume of complete media was added to give a final volume of 200µl. Figure 7-5 shows the GYPC expression and GFP expression of control (GFP expressing) and knockdown cells using flow cytometry at day 18 of culture. No obvious benefit of a higher MOI was seen for either the GYPC knockdown or GFP expression.

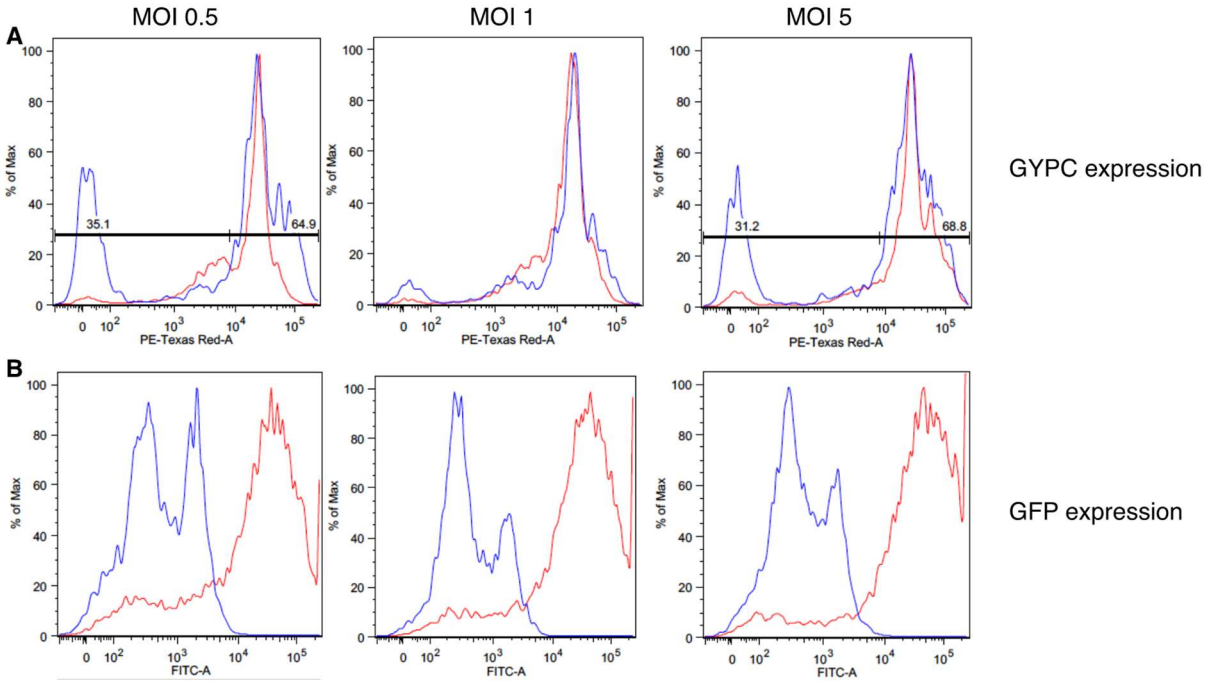


Figure 7-5 Effect of different MOI on GYPC and GFP expression. BLUE- GYPC shRNA transduced cells. RED- control/GFP transduced cells

8 References

ABDI, A., YU, L., GOULDING, D., RONO, M. K., BEJON, P., CHOUDHARY, J. & RAYNER, J. 2017. Proteomic analysis of extracellular vesicles from a. *Wellcome Open Res,* 2**,** 50.

ADAMS, Y., KUHNRAE, P., HIGGINS, M. K., GHUMRA, A. & ROWE, J. A. 2014. Rosetting Plasmodium falciparum-infected erythrocytes bind to human brain microvascular endothelial cells in vitro, demonstrating a dual adhesion phenotype mediated by distinct P. falciparum erythrocyte membrane protein 1 domains. *Infect Immun,* 82**,** 949-59.

AIKAWA, M. 1988. Human cerebral malaria. *Am J Trop Med Hyg,* 39**,** 3-10.

AIRD, W. C., MOSNIER, L. O. & FAIRHURST, R. M. 2014. Plasmodium falciparum picks (on) EPCR. *Blood,* 123**,** 163-7.

AKECH, S., GWER, S., IDRO, R., FEGAN, G., EZIEFULA, A. C., NEWTON, C. R., LEVIN, M. & MAITLAND, K. 2006. Volume expansion with albumin compared to gelofusine in children with severe malaria: results of a controlled trial. *PLoS Clin Trials,* 1**,** e21.

AKECH, S. O., JEMUTAI, J., TIMBWA, M., KIVAYA, E., BOGA, M., FEGAN, G. & MAITLAND, K. 2010. Phase II trial on the use of Dextran 70 or starch for supportive therapy in Kenyan children with severe malaria. *Crit Care Med,* 38**,** 1630-6.

AL-YAMAN, F., GENTON, B., MOKELA, D., RAIKO, A., KATI, S., ROGERSON, S., REEDER, J. & ALPERS, M. 1995. Human cerebral malaria: lack of significant association between erythrocyte rosetting and disease severity. *Trans R Soc Trop Med Hyg,* 89**,** 55-8.

ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K. & WATSON, J. 2014. *Molecular Biology of the Cell,* New York, Garland Science.

ALEY, S. B., BARNWELL, J. W., DANIEL, W. & HOWARD, R. J. 1984. Identification of parasite proteins in a membrane preparation enriched for the surface membrane of erythrocytes infected with Plasmodium knowlesi. *Mol Biochem Parasitol,* 12**,** 69-84.

ALLEN, S. J., O'DONNELL, A., ALEXANDER, N. D., MGONE, C. S., PETO, T. E., CLEGG, J. B., ALPERS, M. P. & WEATHERALL, D. J. 1999. Prevention of cerebral malaria in children in Papua New Guinea by southeast Asian ovalocytosis band 3. *Am J Trop Med Hyg,* 60**,** 1056-60.

ALMUKADI, H., SCHWAKE, C., KAISER, M. M., MAYER, D. C. G., SCHIEMER, J., BALDWIN, M. R., HEGDE, S., LU, Y., HANADA, T. & CHISHTI, A. H. 2019. Human erythrocyte band 3 is a host receptor for. *Blood,* 133**,** 470-480.

ANGELETTI, D., ALBRECHT, L., WAHLGREN, M. & MOLL, K. 2013. Analysis of antibody induction upon immunization with distinct NTS-DBL1α-domains of PfEMP1 from rosetting Plasmodium falciparum parasites. *Malar J,* 12**,** 32.

ANGKASEKWINAI, P., LOOAREESUWAN, S. & CHAIYAROJ, S. C. 1998. Lack of significant association between rosette formation and parasitized erythrocyte adherence to purified CD36. *Southeast Asian J Trop Med Public Health,* 29**,** 41-5.

ANGUS, B. J., THANIKKUL, K., SILAMUT, K., WHITE, N. J. & UDOMSANGPETCH, R. 1996. Short report: Rosette formation in Plasmodium ovale infection. *Am J Trop Med Hyg,* 55**,** 560-1.

ANSTEE, D. J., GAMPEL, A. & TOYE, A. M. 2012. Ex-vivo generation of human red cells for transfusion. *Curr Opin Hematol,* 19**,** 163-9.

ANTONIANI, C., MENEGHINI, V., LATTANZI, A., FELIX, T., ROMANO, O., MAGRIN, E., WEBER, L., PAVANI, G., EL HOSS, S., KURITA, R., NAKAMURA, Y., CRADICK, T. J., LUNDBERG, A. S., PORTEUS, M., AMENDOLA, M., EL NEMER, W., CAVAZZANA, M., MAVILIO, F. & MICCIO, A. 2018. Induction of fetal hemoglobin synthesis by CRISPR/Cas9-mediated editing of the human β-globin locus. *Blood,* 131**,** 1960-1973.

ARAKAWA, T., KOBAYASHI-YURUGI, T., ALGUEL, Y., IWANARI, H., HATAE, H., IWATA, M., ABE, Y., HINO, T., IKEDA-SUNO, C., KUMA, H., KANG, D., MURATA, T., HAMAKUBO, T., CAMERON, A. D., KOBAYASHI, T., HAMASAKI, N. & IWATA, S. 2015. Crystal structure of the anion exchanger domain of human erythrocyte band 3. *Science,* 350**,** 680-4.

ASHLEY, E. A., PYAE PHYO, A. & WOODROW, C. J. 2018. Malaria. *Lancet,* 391**,** 1608-1621.
ATAÍDE, R., MAYOR, A. & ROGERSON, S. J. 2014. Malaria, primigravidae, and antibodies: knowledge gained and future perspectives. *Trends Parasitol,* 30**,** 85-94.
ATKINSON, A., GARNIER, S., AFRIDI, S., FUMOUX, F. & RIHET, P. 2012. Genetic variations in genes involved in heparan sulphate biosynthesis are associated with Plasmodium falciparum parasitaemia: a familial study in Burkina Faso. *Malar J,* 11**,** 108.
ATTAR, A. 2014. Changes in the cell Surface Markers During Normal Hematopoiesis: A Guide to Cell Isolation. *Global Journal of Hematology and Blood Transfusion,* 1**,** 20-28.
AVRIL, M., BERNABEU, M., BENJAMIN, M., BRAZIER, A. J. & SMITH, J. D. 2016. Interaction between Endothelial Protein C Receptor and Intercellular Adhesion Molecule 1 to Mediate Binding of Plasmodium falciparum-Infected Erythrocytes to Endothelial Cells. *MBio,* 7.
AVRIL, M., TRIPATHI, A. K., BRAZIER, A. J., ANDISI, C., JANES, J. H., SOMA, V. L., SULLIVAN, D. J., BULL, P. C., STINS, M. F. & SMITH, J. D. 2012. A restricted subset of var genes mediates adherence of Plasmodium falciparum-infected erythrocytes to brain endothelial cells. *Proc Natl Acad Sci U S A,* 109**,** E1782-90.
AZASI, Y., LINDERGARD, G., GHUMRA, A., MU, J., MILLER, L. H. & ROWE, J. A. 2018. Infected erythrocytes expressing DC13 PfEMP1 differ from recombinant proteins in EPCR-binding function. *Proc Natl Acad Sci U S A,* 115**,** 1063-1068.
BACHMANN, A., PREDEHL, S., MAY, J., HARDER, S., BURCHARD, G. D., GILBERGER, T. W., TANNICH, E. & BRUCHHAUS, I. 2011. Highly co-ordinated var gene expression and switching in clinical Plasmodium falciparum isolates from non-immune malaria patients. *Cell Microbiol,* 13**,** 1397-409.
BACHMANN, A., SCHOLZ, J. A., JANßEN, M., KLINKERT, M. Q., TANNICH, E., BRUCHHAUS, I. & PETTER, M. 2015. A comparative study of the localization and membrane topology of members of the RIFIN, STEVOR and PfMC-2TM protein families in Plasmodium falciparum-infected erythrocytes. *Malar J,* 14**,** 274.
BAI, X. M., VAN DER SCHUEREN, B., CASSIMAN, J. J., VAN DEN BERGHE, H. & DAVID, G. 1994. Differential expression of multiple cell-surface heparan sulfate proteoglycans during embryonic tooth development. *J Histochem Cytochem,* 42**,** 1043-54.
BAND, G., ROCKETT, K. A., SPENCER, C. C., KWIATKOWSKI, D. P. & NETWORK, M. G. E. 2015. A novel locus of resistance to severe malaria in a region of ancient balancing selection. *Nature,* 526**,** 253-7.
BARBER, B. E., GRIGG, M. J., WILLIAM, T., YEO, T. W. & ANSTEY, N. M. 2017. The Treatment of Plasmodium knowlesi Malaria. *Trends Parasitol,* 33**,** 242-253.
BARBER, B. E., WILLIAM, T., GRIGG, M. J., MENON, J., AUBURN, S., MARFURT, J., ANSTEY, N. M. & YEO, T. W. 2013. A prospective comparative study of knowlesi, falciparum, and vivax malaria in Sabah, Malaysia: high proportion with severe disease from Plasmodium knowlesi and Plasmodium vivax but no mortality with early referral and artesunate therapy. *Clin Infect Dis,* 56**,** 383-97.
BARFOD, L., DALGAARD, M. B., PLEMAN, S. T., OFORI, M. F., PLEASS, R. J. & HVIID, L. 2011. Evasion of immunity to Plasmodium falciparum malaria by IgM masking of protective IgG epitopes in infected erythrocyte surface-exposed PfEMP1. *Proc Natl Acad Sci U S A,* 108**,** 12485-90.
BARRAGAN, A., FERNANDEZ, V., CHEN, Q., VON EULER, A., WAHLGREN, M. & SPILLMANN, D. 2000a. The duffy-binding-like domain 1 of Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) is a heparan sulfate ligand that requires 12 mers for binding. *Blood,* 95**,** 3594-9.
BARRAGAN, A., KREMSNER, P. G., WAHLGREN, M. & CARLSON, J. 2000b. Blood group A antigen is a coreceptor in Plasmodium falciparum rosetting. *Infect Immun,* 68**,** 2971-5.
BARRAGAN, A., KREMSNER, P. G., WEISS, W., WAHLGREN, M. & CARLSON, J. 1998. Age-related buildup of humoral immunity against epitopes for rosette formation and agglutination in African areas of malaria endemicity. *Infect Immun,* 66**,** 4783-7.

BARRAGAN, A., SPILLMANN, D., KREMSNER, P. G., WAHLGREN, M. & CARLSON, J. 1999. Plasmodium falciparum: molecular background to strain-specific rosette disruption by glycosaminoglycans and sulfated glycoconjugates. *Exp Parasitol,* 91**,** 133-43.

BARRERA, V., MACCORMICK, I. J. C., CZANNER, G., HISCOTT, P. S., WHITE, V. A., CRAIG, A. G., BEARE, N. A. V., CULSHAW, L. H., ZHENG, Y., BIDDOLPH, S. C., MILNER, D. A., KAMIZA, S., MOLYNEUX, M. E., TAYLOR, T. E. & HARDING, S. P. 2018. Neurovascular sequestration in paediatric. *Elife,* 7.

BARUCH, D. I., GORMELY, J. A., MA, C., HOWARD, R. J. & PASLOSKE, B. L. 1996. Plasmodium falciparum erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc Natl Acad Sci U S A,* 93**,** 3497-502.

BARUCH, D. I., PASLOSKE, B. L., SINGH, H. B., BI, X., MA, X. C., FELDMAN, M., TARASCHI, T. F. & HOWARD, R. J. 1995. Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell,* 82**,** 77-87.

BASTOS, M. F., ALBRECHT, L., KOZLOWSKI, E. O., LOPES, S. C., BLANCO, Y. C., CARLOS, B. C., CASTIÑEIRAS, C., VICENTE, C. P., WERNECK, C. C., WUNDERLICH, G., FERREIRA, M. U., MARINHO, C. R., MOURÃO, P. A., PAVÃO, M. S. & COSTA, F. T. 2014. Fucosylated chondroitin sulfate inhibits Plasmodium falciparum cytoadhesion and merozoite invasion. *Antimicrob Agents Chemother,* 58**,** 1862-71.

BEARE, N. A., HARDING, S. P., TAYLOR, T. E., LEWALLEN, S. & MOLYNEUX, M. E. 2009. Perfusion abnormalities in children with cerebral malaria and malarial retinopathy. *J Infect Dis,* 199**,** 263-71.

BECKMANN, R., SMYTHE, J. S., ANSTEE, D. J. & TANNER, M. J. 1998. Functional cell surface expression of band 3, the human red blood cell anion exchange protein (AE1), in K562 erythroleukemia cells: band 3 enhances the cell surface reactivity of Rh antigens. *Blood,* 92**,** 4428-38.

BEI, A. K., BRUGNARA, C. & DURAISINGH, M. T. 2010. In vitro genetic analysis of an erythrocyte determinant of malaria infection. *J Infect Dis,* 202**,** 1722-7.

BERENDT, A. R., FERGUSON, D. J., GARDNER, J., TURNER, G., ROWE, A., MCCORMICK, C., ROBERTS, D., CRAIG, A., PINCHES, R. & ELFORD, B. C. 1994. Molecular mechanisms of sequestration in malaria. *Parasitology,* 108 Suppl**,** S19-28.

BERTIN, G. I., LAVSTSEN, T., GUILLONNEAU, F., DORITCHAMOU, J., WANG, C. W., JESPERSEN, J. S., EZIMEGNON, S., FIEVET, N., ALAO, M. J., LALYA, F., MASSOUGBODJI, A., NDAM, N. T., THEANDER, T. G. & DELORON, P. 2013. Expression of the domain cassette 8 Plasmodium falciparum erythrocyte membrane protein 1 is associated with cerebral malaria in Benin. *PLoS One,* 8**,** e68368.

BHASIN, V. K. & TRAGER, W. 1984. Gametocyte-forming and non-gametocyte-forming clones of Plasmodium falciparum. *Am J Trop Med Hyg,* 33**,** 534-7.

BOJANG, K. A., PALMER, A., BOELE VAN HENSBROEK, M., BANYA, W. A. & GREENWOOD, B. M. 1997. Management of severe malarial anaemia in Gambian children. *Trans R Soc Trop Med Hyg,* 91**,** 557-61.

BOYLE, M. J., RICHARDS, J. S., GILSON, P. R., CHAI, W. & BEESON, J. G. 2010. Interactions with heparin-like molecules during erythrocyte invasion by Plasmodium falciparum merozoites. *Blood,* 115**,** 4559-68.

BRUCE, L. J., GROVES, J. D., OKUBO, Y., THILAGANATHAN, B. & TANNER, M. J. 1994. Altered band 3 structure and function in glycophorin A- and B-deficient (MkMk) red blood cells. *Blood,* 84**,** 916-22.

BRUCE, L. J., PAN, R. J., COPE, D. L., UCHIKAWA, M., GUNN, R. B., CHERRY, R. J. & TANNER, M. J. 2004. Altered structure and anion transport properties of band 3 (AE1, SLC4A1) in human red cells lacking glycophorin A. *J Biol Chem,* 279**,** 2414-20.

BRUCE, L. J., RING, S. M., ANSTEE, D. J., REID, M. E., WILKINSON, S. & TANNER, M. J. 1995. Changes in the blood group Wright antigens are associated with a mutation at amino acid 658 in human erythrocyte band 3: a site of interaction between band 3 and glycophorin A under certain conditions. *Blood,* 85**,** 541-7.

CABRERA, A., NECULAI, D. & KAIN, K. C. 2014. CD36 and malaria: friends or foes? A decade of data provides some answers. *Trends Parasitol,* 30**,** 436-44.
CAMPONOVO, F., BEVER, C. A., GALACTIONOVA, K., SMITH, T. & PENNY, M. A. 2017. Incidence and admission rates for severe malaria and their impact on mortality in Africa. *Malar J,* 16**,** 1.
CARLSON, J., EKRE, H. P., HELMBY, H., GYSIN, J., GREENWOOD, B. M. & WAHLGREN, M. 1992. Disruption of Plasmodium falciparum erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am J Trop Med Hyg,* 46**,** 595-602.
CARLSON, J., HELMBY, H., HILL, A. V., BREWSTER, D., GREENWOOD, B. M. & WAHLGREN, M. 1990. Human cerebral malaria: association with erythrocyte rosetting and lack of anti-rosetting antibodies. *Lancet,* 336**,** 1457-60.
CARLSON, J., NASH, G. B., GABUTTI, V., AL-YAMAN, F. & WAHLGREN, M. 1994. Natural protection against severe Plasmodium falciparum malaria due to impaired rosette formation. *Blood,* 84**,** 3909-14.
CARLSON, J. & WAHLGREN, M. 1992. Plasmodium falciparum erythrocyte rosetting is mediated by promiscuous lectin-like interactions. *J Exp Med,* 176**,** 1311-7.
CATERSON, B. 2012. Fell-Muir Lecture: chondroitin sulphate glycosaminoglycans: fun for some and confusion for others. *Int J Exp Pathol,* 93**,** 1-10.
CH'NG, J. H., MOLL, K., QUINTANA, M. E. P., CHAN, S. C., MASTERS, E., MOLES, E., LIU, J., ERIKSSON, A. B. & WAHLGREN, M. 2016. Rosette-Disrupting Effect of an Anti-Plasmodial Compound for the Potential Treatment of Plasmodium falciparum Malaria Complications. *Sci Rep,* 6**,** 29317.
CHARUNWATTHANA, P., ABUL FAIZ, M., RUANGVEERAYUT, R., MAUDE, R. J., RAHMAN, M. R., ROBERTS, L. J., MOORE, K., BIN YUNUS, E., HOQUE, M. G., HASAN, M. U., LEE, S. J., PUKRITTAYAKAMEE, S., NEWTON, P. N., WHITE, N. J., DAY, N. P. & DONDORP, A. M. 2009. N-acetylcysteine as adjunctive treatment in severe malaria: a randomized, double-blinded placebo-controlled clinical trial. *Crit Care Med,* 37**,** 516-22.
CHARUNWATTHANA, P., LEITGEB, A., RUEANGVEERAYUTH, R., UTHAISIN, C., SILAMUT, K., SILA, P., LEE, S., LINDGREN, M., MÅNSSON, M., WAHLGREN, M. & DONDORP, A. 2014. Sevuparin: adjunct therapy in P. falciparum malaria shows anti-adhesive and anti-merozoite effectspotentialto improve the outcome of severe malaria. *American Society of Tropical Medicine and Hygiene.* New Orleans, USA.
CHEN, Q., BARRAGAN, A., FERNANDEZ, V., SUNDSTRÖM, A., SCHLICHTHERLE, M., SAHLÉN, A., CARLSON, J., DATTA, S. & WAHLGREN, M. 1998. Identification of Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite P. falciparum. *J Exp Med,* 187**,** 15-23.
CHEN, Q., HEDDINI, A., BARRAGAN, A., FERNANDEZ, V., PEARCE, S. F. & WAHLGREN, M. 2000. The semiconserved head structure of Plasmodium falciparum erythrocyte membrane protein 1 mediates binding to multiple independent host receptors. *J Exp Med,* 192**,** 1-10.
CHEN, Y., GÖTTE, M., LIU, J. & PARK, P. W. 2008. Microbial subversion of heparan sulfate proteoglycans. *Mol Cells,* 26**,** 415-26.
CHOTIVANICH, K., SRITABAL, J., UDOMSANGPETCH, R., NEWTON, P., STEPNIEWSKA, K. A., RUANGVEERAYUTH, R., LOOAREESUWAN, S., ROBERTS, D. J. & WHITE, N. J. 2004. Platelet-induced autoagglutination of Plasmodium falciparum-infected red blood cells and disease severity in Thailand. *J Infect Dis,* 189**,** 1052-5.
CHOTIVANICH, K. T., PUKRITTAYAKAMEE, S., SIMPSON, J. A., WHITE, N. J. & UDOMSANGPETCH, R. 1998. Characteristics of Plasmodium vivax-infected erythrocyte rosettes. *Am J Trop Med Hyg,* 59**,** 73-6.
CHUNG, J. E., MAGIS, W., VU, J., HEO, S. J., WARTIOVAARA, K., WALTERS, M. C., KURITA, R., NAKAMURA, Y., BOFFELLI, D., MARTIN, D. I. K., CORN, J. E. & DEWITT, M. A. 2019. CRISPR-Cas9 interrogation of a putative fetal globin repressor in human erythroid cells. *PLoS One,* 14**,** e0208237.
CLAESSENS, A., ADAMS, Y., GHUMRA, A., LINDERGARD, G., BUCHAN, C. C., ANDISI, C., BULL, P. C., MOK, S., GUPTA, A. P., WANG, C. W., TURNER, L., ARMAN, M., RAZA, A., BOZDECH, Z. & ROWE, J. A. 2012. A subset of group A-like var genes

encodes the malaria parasite ligands for binding to human brain endothelial cells. *Proc Natl Acad Sci U S A,* 109**,** E1772-81.
CLOUGH, B., ATILOLA, F. A., BLACK, J. & PASVOL, G. 1998a. Plasmodium falciparum: the importance of IgM in the rosetting of parasite-infected erythrocytes. *Exp Parasitol,* 89**,** 129-32.
CLOUGH, B., ATILOLA, F. A. & PASVOI, G. 1998b. The role of rosetting in the multiplication of Plasmodium falciparum: rosette formation neither enhances nor targets parasite invasion into uninfected red cells. *Br J Haematol,* 100**,** 99-104.
COCKBURN, I. A., MACKINNON, M. J., O'DONNELL, A., ALLEN, S. J., MOULDS, J. M., BAISOR, M., BOCKARIE, M., REEDER, J. C. & ROWE, J. A. 2004. A human complement receptor 1 polymorphism that reduces Plasmodium falciparum rosetting confers protection against severe malaria. *Proc Natl Acad Sci U S A,* 101**,** 272-7.
CONSTANTINO, B. T. & COGIONIS, B. 2000. Nucleated RBCs—Significance in the Peripheral Blood Film. *Laboratory Medicine,* 31**,** 223-229.
COOLING, L. 2015. Blood Groups in Infection and Host Susceptibility. *Clin Microbiol Rev,* 28**,** 801-70.
COPPI, A., TEWARI, R., BISHOP, J. R., BENNETT, B. L., LAWRENCE, R., ESKO, J. D., BILLKER, O. & SINNIS, P. 2007. Heparan sulfate proteoglycans provide a signal to Plasmodium sporozoites to stop migrating and productively invade host cells. *Cell Host Microbe,* 2**,** 316-27.
CRAIG, A. G., GRAU, G. E., JANSE, C., KAZURA, J. W., MILNER, D., BARNWELL, J. W., TURNER, G., LANGHORNE, J. & MALARIA, P. O. T. H. R. M. O. A. M. F. R. O. S. 2012. The role of animal models for research on severe malaria. *PLoS Pathog,* 8**,** e1002401.
CRANDALL, I., COLLINS, W. E., GYSIN, J. & SHERMAN, I. W. 1993. Synthetic peptides based on motifs present in human band 3 protein inhibit cytoadherence/sequestration of the malaria parasite Plasmodium falciparum. *Proc Natl Acad Sci U S A,* 90**,** 4703-7.
CRAWLEY, J., WARUIRU, C., MITHWANI, S., MWANGI, I., WATKINS, W., OUMA, D., WINSTANLEY, P., PETO, T. & MARSH, K. 2000. Effect of phenobarbital on seizure frequency and mortality in childhood cerebral malaria: a randomised, controlled intervention study. *Lancet,* 355**,** 701-6.
CROSNIER, C., BUSTAMANTE, L. Y., BARTHOLDSON, S. J., BEI, A. K., THERON, M., UCHIKAWA, M., MBOUP, S., NDIR, O., KWIATKOWSKI, D. P., DURAISINGH, M. T., RAYNER, J. C. & WRIGHT, G. J. 2011. Basigin is a receptor essential for erythrocyte invasion by Plasmodium falciparum. *Nature,* 480**,** 534-7.
CSERTI, C. M. & DZIK, W. H. 2007. The ABO blood group system and Plasmodium falciparum malaria. *Blood,* 110**,** 2250-8.
CUMMINGS, R. D. 2009. The repertoire of glycan determinants in the human glycome. *Mol Biosyst,* 5**,** 1087-104.
D'ALESSANDRO, A., RIGHETTI, P. G. & ZOLLA, L. 2010. The red blood cell proteome and interactome: an update. *J Proteome Res,* 9**,** 144-63.
DANKWA, S., CHAAND, M., KANJEE, U., JIANG, R. H. Y., NOBRE, L. V., GOLDBERG, J. M., BEI, A. K., MOECHTAR, M. A., GRÜRING, C., AHOUIDI, A. D., NDIAYE, D., DIEYE, T. N., MBOUP, S., WEEKES, M. P. & DURAISINGH, M. T. 2017. Genetic Evidence for Erythrocyte Receptor Glycophorin B Expression Levels Defining a Dominant Plasmodium falciparum Invasion Pathway into Human Erythrocytes. *Infect Immun,* 85.
DAS, B. K., MISHRA, S., PADHI, P. K., MANISH, R., TRIPATHY, R., SAHOO, P. K. & RAVINDRAN, B. 2003. Pentoxifylline adjunct improves prognosis of human cerebral malaria in adults. *Trop Med Int Health,* 8**,** 680-4.
DAVID, G., BAI, X. M., VAN DER SCHUEREN, B., CASSIMAN, J. J. & VAN DEN BERGHE, H. 1992. Developmental changes in heparan sulfate expression: in situ detection with mAbs. *J Cell Biol,* 119**,** 961-75.
DAVID, P. H., HOMMEL, M., MILLER, L. H., UDEINYA, I. J. & OLIGINO, L. D. 1983. Parasite sequestration in Plasmodium falciparum malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. *Proc Natl Acad Sci U S A,* 80**,** 5075-9.

DAY, N. P., PHU, N. H., MAI, N. T., CHAU, T. T., LOC, P. P., CHUONG, L. V., SINH, D. X., HOLLOWAY, P., HIEN, T. T. & WHITE, N. J. 2000. The pathophysiologic and prognostic significance of acidosis in severe adult malaria. *Crit Care Med,* 28**,** 1833-40.

DEAN, L. 2005. *Blood Groups and Red Cell Antigens,* Bethesda, National Center for Biotechnology Information.

DEANS, A. M., LYKE, K. E., THERA, M. A., PLOWE, C. V., KONÉ, A., DOUMBO, O. K., KAI, O., MARSH, K., MACKINNON, M. J., RAZA, A. & ROWE, J. A. 2006. Low multiplication rates of African Plasmodium falciparum isolates and lack of association of multiplication rate and red blood cell selectivity with malaria virulence. *Am J Trop Med Hyg,* 74**,** 554-63.

DEANS, A. M. & ROWE, J. A. 2006. Plasmodium falciparum: Rosettes do not protect merozoites from invasion-inhibitory antibodies. *Exp Parasitol,* 112**,** 269-73.

DEGAREGE, A., GEBREZGI, M. T., IBANEZ, G., WAHLGREN, M. & MADHIVANAN, P. 2019. Effect of the ABO blood group on susceptibility to severe malaria: A systematic review and meta-analysis. *Blood Rev,* 33**,** 53-62.

DESAI, M., TER KUILE, F. O., NOSTEN, F., MCGREADY, R., ASAMOA, K., BRABIN, B. & NEWMAN, R. D. 2007. Epidemiology and burden of malaria in pregnancy. *Lancet Infect Dis,* 7**,** 93-104.

DESAI, U. R., WANG, H. M. & LINHARDT, R. J. 1993. Specificity studies on the heparin lyases from Flavobacterium heparinum. *Biochemistry,* 32**,** 8140-5.

DI PERRI, G., DI PERRI, I. G., MONTEIRO, G. B., BONORA, S., HENNIG, C., CASSATELLA, M., MICCIOLO, R., VENTO, S., DUSI, S. & BASSETTI, D. 1995. Pentoxifylline as a supportive agent in the treatment of cerebral malaria in children. *J Infect Dis,* 171**,** 1317-22.

DONDORP, A. M., FANELLO, C. I., HENDRIKSEN, I. C., GOMES, E., SENI, A., CHHAGANLAL, K. D., BOJANG, K., OLAOSEBIKAN, R., ANUNOBI, N., MAITLAND, K., KIVAYA, E., AGBENYEGA, T., NGUAH, S. B., EVANS, J., GESASE, S., KAHABUKA, C., MTOVE, G., NADJM, B., DEEN, J., MWANGA-AMUMPAIRE, J., NANSUMBA, M., KAREMA, C., UMULISA, N., UWIMANA, A., MOKUOLU, O. A., ADEDOYIN, O. T., JOHNSON, W. B., TSHEFU, A. K., ONYAMBOKO, M. A., SAKULTHAEW, T., NGUM, W. P., SILAMUT, K., STEPNIEWSKA, K., WOODROW, C. J., BETHELL, D., WILLS, B., ONEKO, M., PETO, T. E., VON SEIDLEIN, L., DAY, N. P., WHITE, N. J. & GROUP, A. 2010. Artesunate versus quinine in the treatment of severe falciparum malaria in African children (AQUAMAT): an open-label, randomised trial. *Lancet,* 376**,** 1647-57.

DONDORP, A. M., INCE, C., CHARUNWATTHANA, P., HANSON, J., VAN KUIJEN, A., FAIZ, M. A., RAHMAN, M. R., HASAN, M., BIN YUNUS, E., GHOSE, A., RUANGVEERAYUT, R., LIMMATHUROTSAKUL, D., MATHURA, K., WHITE, N. J. & DAY, N. P. 2008a. Direct in vivo assessment of microcirculatory dysfunction in severe falciparum malaria. *J Infect Dis,* 197**,** 79-84.

DONDORP, A. M., LEE, S. J., FAIZ, M. A., MISHRA, S., PRICE, R., TJITRA, E., THAN, M., HTUT, Y., MOHANTY, S., YUNUS, E. B., RAHMAN, R., NOSTEN, F., ANSTEY, N. M., DAY, N. P. & WHITE, N. J. 2008b. The relationship between age and the manifestations of and mortality associated with severe malaria. *Clin Infect Dis,* 47**,** 151-7.

DONDORP, A. M., PONGPONRATN, E. & WHITE, N. J. 2004. Reduced microcirculatory flow in severe falciparum malaria: pathophysiology and electron-microscopic pathology. *Acta Trop,* 89**,** 309-17.

DONDORP, A. M., SILAMUT, K., CHARUNWATTHANA, P., CHUASUWANCHAI, S., RUANGVEERAYUT, R., KRINTRATUN, S., WHITE, N. J., HO, M. & DAY, N. P. 2007. Levamisole inhibits sequestration of infected red blood cells in patients with falciparum malaria. *J Infect Dis,* 196**,** 460-6.

DONG, W., LU, W., MCKEEHAN, W. L., LUO, Y. & YE, S. 2012. Structural basis of heparan sulfate-specific degradation by heparinase III. *Protein Cell,* 3**,** 950-61.

DOUDNA, J. A. & CHARPENTIER, E. 2014. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science,* 346**,** 1258096.

DOUMBO, O. K., THERA, M. A., KONÉ, A. K., RAZA, A., TEMPEST, L. J., LYKE, K. E., PLOWE, C. V. & ROWE, J. A. 2009. High levels of Plasmodium falciparum rosetting in all clinical forms of severe malaria in African children. *Am J Trop Med Hyg,* 81**,** 987-93.

DREXLER, H., DIRKS, W., MACLEOD, R., QUENTMEIER, H., STEUBE, K. & UPHOFF, C. 2001. DSMZ Catalogue of human and animal cell cultures. 8th ed. Germany: Braunschweig.

DUNDAS, K., SHEARS, M. J., SINNIS, P. & WRIGHT, G. J. 2018. Important Extracellular Interactions between Plasmodium Sporozoites and Host Cells Required for Infection. *Trends Parasitol*.

EASTERBROOK, J., FIDANZA, A. & FORRESTER, L. M. 2016. Concise review: programming human pluripotent stem cells into blood. *Br J Haematol,* 173**,** 671-9.

EDA, S., LAWLER, J. & SHERMAN, I. W. 1999. Plasmodium falciparum-infected erythrocyte adhesion to the type 3 repeat domain of thrombospondin-1 is mediated by a modified band 3 protein. *Mol Biochem Parasitol,* 100**,** 195-205.

EGAN, E. S., JIANG, R. H., MOECHTAR, M. A., BARTENEVA, N. S., WEEKES, M. P., NOBRE, L. V., GYGI, S. P., PAULO, J. A., FRANTZREB, C., TANI, Y., TAKAHASHI, J., WATANABE, S., GOLDBERG, J., PAUL, A. S., BRUGNARA, C., ROOT, D. E., WIEGAND, R. C., DOENCH, J. G. & DURAISINGH, M. T. 2015. Malaria. A forward genetic screen identifies erythrocyte CD55 as essential for Plasmodium falciparum invasion. *Science,* 348**,** 711-4.

FALK, N., KAESTLI, M., QI, W., OTT, M., BAEA, K., CORTÉS, A. & BECK, H. P. 2009. Analysis of Plasmodium falciparum var genes expressed in children from Papua New Guinea. *J Infect Dis,* 200**,** 347-56.

FERREIRA, A. F., CALIN, G. A., PICANÇO-CASTRO, V., KASHIMA, S., COVAS, D. T. & DE CASTRO, F. A. 2018. Hematopoietic stem cells from induced pluripotent stem cells - considering the role of microRNA as a cell differentiation regulator. *J Cell Sci,* 131.

FIELD, S. P., HEMPELMANN, E., MENDELOW, B. V. & FLEMING, A. F. 1994. Glycophorin variants and Plasmodium falciparum: protective effect of the Dantu phenotype in vitro. *Hum Genet,* 93**,** 148-50.

FIGUEROA, D. 2013. The Diego blood group system: a review. *Immunohematology,* 29**,** 73-81.

FOQUET, L., SCHAFER, C., MINKAH, N. K., ALANINE, D. G. W., FLANNERY, E. L., STEEL, R. W. J., SACK, B. K., CAMARGO, N., FISHBAUGHER, M., BETZ, W., NGUYEN, T., BILLMAN, Z. P., WILSON, E. M., BIAL, J., MURPHY, S. C., DRAPER, S. J., MIKOLAJCZAK, S. A. & KAPPE, S. H. I. 2018. Liver Stage Infection and Transition to Stable Blood Stage Infection in Liver-Humanized and Blood-Humanized FRGN KO Mice Enables Testing of Blood Stage Inhibitory Antibodies (Reticulocyte-Binding Protein Homolog 5). *Front Immunol,* 9**,** 524.

FREVERT, U., SINNIS, P., CERAMI, C., SHREFFLER, W., TAKACS, B. & NUSSENZWEIG, V. 1993. Malaria circumsporozoite protein binds to heparan sulfate proteoglycans associated with the surface membrane of hepatocytes. *J Exp Med,* 177**,** 1287-98.

FRY, A. E., GHANSA, A., SMALL, K. S., PALMA, A., AUBURN, S., DIAKITE, M., GREEN, A., CAMPINO, S., TEO, Y. Y., CLARK, T. G., JEFFREYS, A. E., WILSON, J., JALLOW, M., SISAY-JOOF, F., PINDER, M., GRIFFITHS, M. J., PESHU, N., WILLIAMS, T. N., NEWTON, C. R., MARSH, K., MOLYNEUX, M. E., TAYLOR, T. E., KORAM, K. A., ODURO, A. R., ROGERS, W. O., ROCKETT, K. A., SABETI, P. C. & KWIATKOWSKI, D. P. 2009. Positive selection of a CD36 nonsense variant in sub-Saharan Africa, but no association with severe malaria phenotypes. *Hum Mol Genet,* 18**,** 2683-92.

GANDHI, N. S. & MANCERA, R. L. 2008. The structure of glycosaminoglycans and their interactions with proteins. *Chem Biol Drug Des,* 72**,** 455-82.

GARDNER, M. J., HALL, N., FUNG, E., WHITE, O., BERRIMAN, M., HYMAN, R. W., CARLTON, J. M., PAIN, A., NELSON, K. E., BOWMAN, S., PAULSEN, I. T., JAMES, K., EISEN, J. A., RUTHERFORD, K., SALZBERG, S. L., CRAIG, A., KYES, S., CHAN, M. S., NENE, V., SHALLOM, S. J., SUH, B., PETERSON, J., ANGIUOLI, S., PERTEA, M., ALLEN, J., SELENGUT, J., HAFT, D., MATHER, M. W., VAIDYA,

A. B., MARTIN, D. M., FAIRLAMB, A. H., FRAUNHOLZ, M. J., ROOS, D. S., RALPH, S. A., MCFADDEN, G. I., CUMMINGS, L. M., SUBRAMANIAN, G. M., MUNGALL, C., VENTER, J. C., CARUCCI, D. J., HOFFMAN, S. L., NEWBOLD, C., DAVIS, R. W., FRASER, C. M. & BARRELL, B. 2002. Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature,* 419**,** 498-511.

GARG, S., AGARWAL, S., DABRAL, S., KUMAR, N., SEHRAWAT, S. & SINGH, S. 2015. Visualization and quantification of Plasmodium falciparum intraerythrocytic merozoites. *Syst Synth Biol,* 9**,** 23-6.

GAUTIER, E. F., DUCAMP, S., LEDUC, M., SALNOT, V., GUILLONNEAU, F., DUSSIOT, M., HALE, J., GIARRATANA, M. C., RAIMBAULT, A., DOUAY, L., LACOMBE, C., MOHANDAS, N., VERDIER, F., ZERMATI, Y. & MAYEUX, P. 2016. Comprehensive Proteomic Analysis of Human Erythropoiesis. *Cell Rep,* 16**,** 1470-1484.

GENTON, B., AL-YAMAN, F., MGONE, C. S., ALEXANDER, N., PANIU, M. M., ALPERS, M. P. & MOKELA, D. 1995. Ovalocytosis and cerebral malaria. *Nature,* 378**,** 564-5.

GHUMRA, A., KHUNRAE, P., ATAIDE, R., RAZA, A., ROGERSON, S. J., HIGGINS, M. K. & ROWE, J. A. 2011. Immunisation with recombinant PfEMP1 domains elicits functional rosette-inhibiting and phagocytosis-inducing antibodies to Plasmodium falciparum. *PLoS One,* 6**,** e16414.

GHUMRA, A., SEMBLAT, J. P., ATAIDE, R., KIFUDE, C., ADAMS, Y., CLAESSENS, A., ANONG, D. N., BULL, P. C., FENNELL, C., ARMAN, M., AMAMBUA-NGWA, A., WALTHER, M., CONWAY, D. J., KASSAMBARA, L., DOUMBO, O. K., RAZA, A. & ROWE, J. A. 2012. Induction of strain-transcending antibodies against Group A PfEMP1 surface antigens from virulent malaria parasites. *PLoS Pathog,* 8**,** e1002665.

GHUMRA, A., SEMBLAT, J. P., MCINTOSH, R. S., RAZA, A., RASMUSSEN, I. B., BRAATHEN, R., JOHANSEN, F. E., SANDLIE, I., MONGINI, P. K., ROWE, J. A. & PLEASS, R. J. 2008. Identification of residues in the Cmu4 domain of polymeric IgM essential for interaction with Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1). *J Immunol,* 181**,** 1988-2000.

GIARRATANA, M. C., KOBARI, L., LAPILLONNE, H., CHALMERS, D., KIGER, L., CYNOBER, T., MARDEN, M. C., WAJCMAN, H. & DOUAY, L. 2005. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nat Biotechnol,* 23**,** 69-74.

GIARRATANA, M. C., ROUARD, H., DUMONT, A., KIGER, L., SAFEUKUI, I., LE PENNEC, P. Y., FRANÇOIS, S., TRUGNAN, G., PEYRARD, T., MARIE, T., JOLLY, S., HEBERT, N., MAZURIER, C., MARIO, N., HARMAND, L., LAPILLONNE, H., DEVAUX, J. Y. & DOUAY, L. 2011. Proof of principle for transfusion of in vitro-generated red blood cells. *Blood,* 118**,** 5071-9.

GOEL, S., PALMKVIST, M., MOLL, K., JOANNIN, N., LARA, P., AKHOURI, R. R., MORADI, N., ÖJEMALM, K., WESTMAN, M., ANGELETTI, D., KJELLIN, H., LEHTIÖ, J., BLIXT, O., IDESTRÖM, L., GAHMBERG, C. G., STORRY, J. R., HULT, A. K., OLSSON, M. L., VON HEIJNE, G., NILSSON, I. & WAHLGREN, M. 2015. RIFINs are adhesins implicated in severe Plasmodium falciparum malaria. *Nat Med,* 21**,** 314-7.

GOEL, V. K., LI, X., CHEN, H., LIU, S. C., CHISHTI, A. H. & OH, S. S. 2003. Band 3 is a host receptor binding merozoite surface protein 1 during the Plasmodium falciparum invasion of erythrocytes. *Proc Natl Acad Sci U S A,* 100**,** 5164-9.

GONÇALVES, B. P., HUANG, C. Y., MORRISON, R., HOLTE, S., KABYEMELA, E., PREVOTS, D. R., FRIED, M. & DUFFY, P. E. 2014. Parasite burden and severity of malaria in Tanzanian children. *N Engl J Med,* 370**,** 1799-808.

GOWD, V. & NANDINI, C. D. 2015. Erythrocytes in the combined milieu of high glucose and high cholesterol shows glycosaminoglycan-dependent cytoadherence to extracellular matrix components. *Int J Biol Macromol,* 73**,** 182-8.

GROVER, A., MANCINI, E., MOORE, S., MEAD, A. J., ATKINSON, D., RASMUSSEN, K. D., O'CARROLL, D., JACOBSEN, S. E. & NERLOV, C. 2014. Erythropoietin guides multipotent hematopoietic progenitor cells toward an erythroid fate. *J Exp Med,* 211**,** 181-8.

GROVES, J., RING, S., SCHOFIELD, A. & TANNER, M. 1983. The expression of the abnormal human red cell anion transporter from South- East

Asian ovalocytes (band 3 SAO) in Xenopus oocytes. FEBS letter.
GROVES, J. D., RING, S. M., SCHOFIELD, A. E. & TANNER, M. J. 1993. The expression of the abnormal human red cell anion transporter from South-East Asian ovalocytes (band 3 SAO) in Xenopus oocytes. *FEBS Lett,* 330**,** 186-90.
GROVES, J. D. & TANNER, M. J. 1992. Glycophorin A facilitates the expression of human band 3-mediated anion transport in Xenopus oocytes. *J Biol Chem,* 267**,** 22163-70.
GUILLOTTE, M., JUILLERAT, A., IGONET, S., HESSEL, A., PETRES, S., CRUBLET, E., LE SCANF, C., LEWIT-BENTLEY, A., BENTLEY, G. A., VIGAN-WOMAS, I. & MERCEREAU-PUIJALON, O. 2015. Immunogenicity of the Plasmodium falciparum PfEMP1-VarO Adhesin: Induction of Surface-Reactive and Rosette-Disrupting Antibodies to VarO Infected Erythrocytes. *PLoS One,* 10**,** e0134292.
GUILLOTTE, M., NATO, F., JUILLERAT, A., HESSEL, A., MARCHAND, F., LEWIT-BENTLEY, A., BENTLEY, G. A., VIGAN-WOMAS, I. & MERCEREAU-PUIJALON, O. 2016. Functional analysis of monoclonal antibodies against the Plasmodium falciparum PfEMP1-VarO adhesin. *Malar J,* 15**,** 28.
GUO, J., WANG, W., YU, D. & WU, Y. 2011. Spinoculation triggers dynamic actin and cofilin activity that facilitates HIV-1 infection of transformed and resting CD4 T cells. *J Virol,* 85**,** 9824-33.
GUZNICZAK, E., MOHAMMAD ZADEH, M., DEMPSEY, F., JIMENEZ, M., BOCK, H., WHYTE, G., WILLOUGHBY, N. & BRIDLE, H. 2017. High-throughput assessment of mechanical properties of stem cell derived red blood cells, toward cellular downstream processing. *Sci Rep,* 7**,** 14457.
GWER, S. A., IDRO, R. I., FEGAN, G., CHENGO, E. M., MPOYA, A., KIVAYA, E., CRAWLEY, J., MUCHOHI, S. N., KIHARA, M. N., OGUTU, B. R., KIRKHAM, F. J. & NEWTON, C. R. 2013. Fosphenytoin for seizure prevention in childhood coma in Africa: a randomized clinical trial. *J Crit Care,* 28**,** 1086-92.
HANDUNETTI, S., GILLADOGA, A. & HOWARD, R. 1990. *A new cytoadherence property of Plasmodium falciparum-infected erythrocytes: Rosetting with uninfected erythrocytes, in Cellular and Molecular Biology of Normal and Abnormal Erythroid Membranes.,* New York, AR Liss.
HANDUNNETTI, S. M., GILLADOGA, A. D., VAN SCHRAVENDIJK, M. R., NAKAMURA, K., AIKAWA, M. & HOWARD, R. J. 1992a. Purification and in vitro selection of rosette-positive (R+) and rosette-negative (R-) phenotypes of knob-positive Plasmodium falciparum parasites. *Am J Trop Med Hyg,* 46**,** 371-81.
HANDUNNETTI, S. M., VAN SCHRAVENDIJK, M. R., HASLER, T., BARNWELL, J. W., GREENWALT, D. E. & HOWARD, R. J. 1992b. Involvement of CD36 on erythrocytes as a rosetting receptor for Plasmodium falciparum-infected erythrocytes. *Blood,* 80**,** 2097-104.
HAWKSWORTH, J., SATCHWELL, T. J., MEINDERS, M., DANIELS, D. E., REGAN, F., THORNTON, N. M., WILSON, M. C., DOBBE, J. G., STREEKSTRA, G. J., TRAKARNSANGA, K., HEESOM, K. J., ANSTEE, D. J., FRAYNE, J. & TOYE, A. M. 2018. Enhancement of red blood cell transfusion compatibility using CRISPR-mediated erythroblast gene editing. *EMBO Mol Med,* 10.
HEDDINI, A., PETTERSSON, F., KAI, O., SHAFI, J., OBIERO, J., CHEN, Q., BARRAGAN, A., WAHLGREN, M. & MARSH, K. 2001. Fresh isolates from children with severe Plasmodium falciparum malaria bind to multiple receptors. *Infect Immun,* 69**,** 5849-56.
HEGEDŰS, T., CHAUBEY, P. M., VÁRADY, G., SZABÓ, E., SARANKÓ, H., HOFSTETTER, L., ROSCHITZKI, B., STIEGER, B. & SARKADI, B. 2015. Inconsistencies in the red blood cell membrane proteome analysis: generation of a database for research and diagnostic applications. *Database (Oxford),* 2015**,** bav056.
HEMMER, C. J., KERN, P., HOLST, F. G., NAWROTH, P. P. & DIETRICH, M. 1991. Neither heparin nor acetylsalicylic acid influence the clinical course in human Plasmodium falciparum malaria: a prospective randomized study. *Am J Trop Med Hyg,* 45**,** 608-12.

HERMENTIN, P., DAVID, P. H., MILLER, L. H., PERKINS, M. E., PASVOL, G. & DAHR, W. 1985. Wright (a + b-) human erythrocytes and Plasmodium falciparum malaria. *Blut,* 50**,** 75-80.
HOFFMAN, S. L., RUSTAMA, D., PUNJABI, N. H., SURAMPAET, B., SANJAYA, B., DIMPUDUS, A. J., MCKEE, K. T., PALEOLOGO, F. P., CAMPBELL, J. R. & MARWOTO, H. 1988. High-dose dexamethasone in quinine-treated patients with cerebral malaria: a double-blind, placebo-controlled trial. *J Infect Dis,* 158**,** 325-31.
HORROCKS, P., PINCHES, R. A., CHAKRAVORTY, S. J., PAPAKRIVOS, J., CHRISTODOULOU, Z., KYES, S. A., URBAN, B. C., FERGUSON, D. J. & NEWBOLD, C. I. 2005. PfEMP1 expression is reduced on the surface of knobless Plasmodium falciparum infected erythrocytes. *J Cell Sci,* 118**,** 2507-18.
HU, J., LIU, J., XUE, F., HALVERSON, G., REID, M., GUO, A., CHEN, L., RAZA, A., GALILI, N., JAFFRAY, J., LANE, J., CHASIS, J. A., TAYLOR, N., MOHANDAS, N. & AN, X. 2013. Isolation and functional characterization of human erythroblasts at distinct stages: implications for understanding of normal and disordered erythropoiesis in vivo. *Blood,* 121**,** 3246-53.
HU, X. 2016. CRISPR/Cas9 system and its applications in human hematopoietic cells. *Blood Cells Mol Dis,* 62**,** 6-12.
HUANG, C. H., REID, M. E., XIE, S. S. & BLUMENFELD, O. O. 1996. Human red blood cell Wright antigens: a genetic and evolutionary perspective on glycophorin A-band 3 interaction. *Blood,* 87**,** 3942-7.
HUGHES, K. R., BIAGINI, G. A. & CRAIG, A. G. 2010. Continued cytoadherence of Plasmodium falciparum infected red blood cells after antimalarial treatment. *Mol Biochem Parasitol,* 169**,** 71-8.
HVIID, L. & JENSEN, A. T. 2015. PfEMP1 - A Parasite Protein Family of Key Importance in Plasmodium falciparum Malaria Immunity and Pathogenesis. *Adv Parasitol,* 88**,** 51-84.
IDRO, R., MARSH, K., JOHN, C. C. & NEWTON, C. R. 2010. Cerebral malaria: mechanisms of brain injury and strategies for improved neurocognitive outcome. *Pediatr Res,* 68**,** 267-74.
IOZZO, R. V. & SCHAEFER, L. 2015. Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix Biol,* 42**,** 11-55.
ISSITT, P. D., PAVONE, B. G., WAGSTAFF, W. & GOLDFINGER, D. 1976. The phenotypes En(a-), Wr(a-b-), and En(a+), Wr(a+b-), and further studies on the Wright and En blood group systems. *Transfusion,* 16**,** 396-407.
JACKSON, M., MA, R., TAYLOR, A. H., AXTON, R. A., EASTERBROOK, J., KYDONAKI, M., OLIVIER, E., MARENAH, L., STANLEY, E. G., ELEFANTY, A. G., MOUNTFORD, J. C. & FORRESTER, L. M. 2016. Enforced Expression of HOXB4 in Human Embryonic Stem Cells Enhances the Production of Hematopoietic Progenitors but Has No Effect on the Maturation of Red Blood Cells. *Stem Cells Transl Med,* 5**,** 981-90.
JASKIEWICZ, E., PEYRARD, T., KACZMAREK, R., ZERKA, A., JODLOWSKA, M. & CZERWINSKI, M. 2018. The Gerbich blood group system: old knowledge, new importance. *Transfus Med Rev,* 32**,** 111-116.
JESPERSEN, J. S., WANG, C. W., MKUMBAYE, S. I., MINJA, D. T., PETERSEN, B., TURNER, L., PETERSEN, J. E., LUSINGU, J. P., THEANDER, T. G. & LAVSTSEN, T. 2016. Plasmodium falciparum var genes expressed in children with severe malaria encode CIDRα1 domains. *EMBO Mol Med,* 8**,** 839-50.
JINEK, M., CHYLINSKI, K., FONFARA, I., HAUER, M., DOUDNA, J. A. & CHARPENTIER, E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science,* 337**,** 816-21.
JOERGENSEN, L., BENGTSSON, D. C., BENGTSSON, A., RONANDER, E., BERGER, S. S., TURNER, L., DALGAARD, M. B., CHAM, G. K., VICTOR, M. E., LAVSTSEN, T., THEANDER, T. G., ARNOT, D. E. & JENSEN, A. T. 2010. Surface co-expression of two different PfEMP1 antigens on single plasmodium falciparum-infected erythrocytes facilitates binding to ICAM1 and PECAM1. *PLoS Pathog,* 6**,** e1001083.
JUILLERAT, A., LEWIT-BENTLEY, A., GUILLOTTE, M., GANGNARD, S., HESSEL, A., BARON, B., VIGAN-WOMAS, I., ENGLAND, P., MERCEREAU-PUIJALON, O. &

BENTLEY, G. A. 2011. Structure of a Plasmodium falciparum PfEMP1 rosetting domain reveals a role for the N-terminal segment in heparin-mediated rosette inhibition. *Proc Natl Acad Sci U S A,* 108**,** 5243-8.
KAESTLI, M., COCKBURN, I. A., CORTÉS, A., BAEA, K., ROWE, J. A. & BECK, H. P. 2006. Virulence of malaria is associated with differential expression of Plasmodium falciparum var gene subgroups in a case-control study. *J Infect Dis,* 193**,** 1567-74.
KALLI, A. C. & REITHMEIER, R. A. F. 2018. Interaction of the human erythrocyte Band 3 anion exchanger 1 (AE1, SLC4A1) with lipids and glycophorin A: Molecular organization of the Wright (Wr) blood group antigen. *PLoS Comput Biol,* 14**,** e1006284.
KAMHI, E., JOO, E. J., DORDICK, J. S. & LINHARDT, R. J. 2013. Glycosaminoglycans in infectious disease. *Biol Rev Camb Philos Soc,* 88**,** 928-43.
KANJEE, U., GRÜRING, C., CHAAND, M., LIN, K. M., EGAN, E., MANZO, J., JONES, P. L., YU, T., BARKER, R., WEEKES, M. P. & DURAISINGH, M. T. 2017. CRISPR/Cas9 knockouts reveal genetic interaction between strain-transcendent erythrocyte determinants of. *Proc Natl Acad Sci U S A,* 114**,** E9356-E9365.
KAUL, D. K., ROTH, E. F., NAGEL, R. L., HOWARD, R. J. & HANDUNNETTI, S. M. 1991. Rosetting of Plasmodium falciparum-infected red blood cells with uninfected red blood cells enhances microvascular obstruction under flow conditions. *Blood,* 78**,** 812-9.
KILEJIAN, A. 1979. Characterization of a protein correlated with the production of knob-like protrusions on membranes of erythrocytes infected with Plasmodium falciparum. *Proc Natl Acad Sci U S A,* 76**,** 4650-3.
KOBAYASHI, K., KATO, K., SUGI, T., TAKEMAE, H., PANDEY, K., GONG, H., TOHYA, Y. & AKASHI, H. 2010. Plasmodium falciparum BAEBL binds to heparan sulfate proteoglycans on the human erythrocyte surface. *J Biol Chem,* 285**,** 1716-25.
KRAEMER, S. M. & SMITH, J. D. 2006. A family affair: var genes, PfEMP1 binding, and malaria disease. *Curr Opin Microbiol,* 9**,** 374-80.
KUN, J. F., SCHMIDT-OTT, R. J., LEHMAN, L. G., LELL, B., LUCKNER, D., GREVE, B., MATOUSEK, P. & KREMSNER, P. G. 1998. Merozoite surface antigen 1 and 2 genotypes and rosetting of Plasmodium falciparum in severe and mild malaria in Lambaréné, Gabon. *Trans R Soc Trop Med Hyg,* 92**,** 110-4.
KURITA, R., SUDA, N., SUDO, K., MIHARADA, K., HIROYAMA, T., MIYOSHI, H., TANI, K. & NAKAMURA, Y. 2013. Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells. *PLoS One,* 8**,** e59890.
KYES, S. A., ROWE, J. A., KRIEK, N. & NEWBOLD, C. I. 1999. Rifins: a second family of clonally variant proteins expressed on the surface of red cells infected with Plasmodium falciparum. *Proc Natl Acad Sci U S A,* 96**,** 9333-8.
KYRIACOU, H. M., STEEN, K. E., RAZA, A., ARMAN, M., WARIMWE, G., BULL, P. C., HAVLIK, I. & ROWE, J. A. 2007. In vitro inhibition of Plasmodium falciparum rosette formation by Curdlan sulfate. *Antimicrob Agents Chemother,* 51**,** 1321-6.
KYRIACOU, H. M., STONE, G. N., CHALLIS, R. J., RAZA, A., LYKE, K. E., THERA, M. A., KONÉ, A. K., DOUMBO, O. K., PLOWE, C. V. & ROWE, J. A. 2006. Differential var gene transcription in Plasmodium falciparum isolates from patients with cerebral malaria compared to hyperparasitaemia. *Mol Biochem Parasitol,* 150**,** 211-8.
LAMBROS, C. & VANDERBERG, J. P. 1979. Synchronization of Plasmodium falciparum erythrocytic stages in culture. *J Parasitol,* 65**,** 418-20.
LANGFITT, J. T., MCDERMOTT, M. P., BRIM, R., MBOMA, S., POTCHEN, M. J., KAMPONDENI, S. D., SEYDEL, K. B., SEMRUD-CLIKEMAN, M. & TAYLOR, T. E. 2019. Neurodevelopmental Impairments 1 Year After Cerebral Malaria. *Pediatrics,* 143.
LAPILLONNE, H., KOBARI, L., MAZURIER, C., TROPEL, P., GIARRATANA, M. C., ZANELLA-CLEON, I., KIGER, L., WATTENHOFER-DONZÉ, M., PUCCIO, H., HEBERT, N., FRANCINA, A., ANDREU, G., VIVILLE, S. & DOUAY, L. 2010. Red blood cell generation from human induced pluripotent stem cells: perspectives for transfusion medicine. *Haematologica,* 95**,** 1651-9.

LAVSTSEN, T., SALANTI, A., JENSEN, A. T., ARNOT, D. E. & THEANDER, T. G. 2003. Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. *Malar J,* 2**,** 27.

LAVSTSEN, T., TURNER, L., SAGUTI, F., MAGISTRADO, P., RASK, T. S., JESPERSEN, J. S., WANG, C. W., BERGER, S. S., BARAKA, V., MARQUARD, A. M., SEGUIN-ORLANDO, A., WILLERSLEV, E., GILBERT, M. T., LUSINGU, J. & THEANDER, T. G. 2012. Plasmodium falciparum erythrocyte membrane protein 1 domain cassettes 8 and 13 are associated with severe malaria in children. *Proc Natl Acad Sci U S A,* 109**,** E1791-800.

LAZAR, H. L., BOKESCH, P. M., VAN LENTA, F., FITZGERALD, C., EMMETT, C., MARSH, H. C., RYAN, U. & GROUP, O. A. T. T. C. S. S. 2004. Soluble human complement receptor 1 limits ischemic damage in cardiac surgery patients at high risk requiring cardiopulmonary bypass. *Circulation,* 110**,** II274-9.

LE SCANF, C., VIGAN-WOMAS, I., CONTAMIN, H., GUILLOTTE, M., BISCHOFF, E. & MERCEREAU-PUIJALON, O. 2008. Rosetting is associated with increased Plasmodium falciparum in vivo multiplication rate in the Saimiri sciureus monkey. *Microbes Infect,* 10**,** 447-51.

LEE, W. C., MALLERET, B., LAU, Y. L., MAUDUIT, M., FONG, M. Y., CHO, J. S., SUWANARUSK, R., ZHANG, R., ALBRECHT, L., COSTA, F. T., PREISER, P., MCGREADY, R., RENIA, L., NOSTEN, F. & RUSSELL, B. 2014. Glycophorin C (CD236R) mediates vivax malaria parasite rosetting to normocytes. *Blood,* 123**,** e100-9.

LEFFLER, E. M., BAND, G., BUSBY, G. B. J., KIVINEN, K., LE, Q. S., CLARKE, G. M., BOJANG, K. A., CONWAY, D. J., JALLOW, M., SISAY-JOOF, F., BOUGOUMA, E. C., MANGANO, V. D., MODIANO, D., SIRIMA, S. B., ACHIDI, E., APINJOH, T. O., MARSH, K., NDILA, C. M., PESHU, N., WILLIAMS, T. N., DRAKELEY, C., MANJURANO, A., REYBURN, H., RILEY, E., KACHALA, D., MOLYNEUX, M., NYIRONGO, V., TAYLOR, T., THORNTON, N., TILLEY, L., GRIMSLEY, S., DRURY, E., STALKER, J., CORNELIUS, V., HUBBART, C., JEFFREYS, A. E., ROWLANDS, K., ROCKETT, K. A., SPENCER, C. C. A., KWIATKOWSKI, D. P. & NETWORK, M. G. E. 2017. Resistance to malaria through structural variation of red blood cell invasion receptors. *Science,* 356.

LEITGEB, A. M., BLOMQVIST, K., CHO-NGWA, F., SAMJE, M., NDE, P., TITANJI, V. & WAHLGREN, M. 2011. Low anticoagulant heparin disrupts Plasmodium falciparum rosettes in fresh clinical isolates. *Am J Trop Med Hyg,* 84**,** 390-6.

LEITGEB, A. M., CHARUNWATTHANA, P., RUEANGVEERAYUT, R., UTHAISIN, C., SILAMUT, K., CHOTIVANICH, K., SILA, P., MOLL, K., LEE, S. J., LINDGREN, M., HOLMER, E., FÄRNERT, A., KIWUWA, M. S., KRISTENSEN, J., HERDER, C., TARNING, J., WAHLGREN, M. & DONDORP, A. M. 2017. Inhibition of merozoite invasion and transient de-sequestration by sevuparin in humans with Plasmodium falciparum malaria. *PLoS One,* 12**,** e0188754.

LELL, B., KÖHLER, C., WAMOLA, B., OLOLA, C. H., KIVAYA, E., KOKWARO, G., WYPIJ, D., MITHWANI, S., TAYLOR, T. E., KREMSNER, P. G. & NEWTON, C. R. 2010. Pentoxifylline as an adjunct therapy in children with cerebral malaria. *Malar J,* 9**,** 368.

LOOAREESUWAN, S., SJOSTROM, L., KRUDSOOD, S., WILAIRATANA, P., PORTER, R. S., HILLS, F. & WARRELL, D. A. 1999. Polyclonal anti-tumor necrosis factor-alpha Fab used as an ancillary treatment for severe malaria. *Am J Trop Med Hyg,* 61**,** 26-33.

LOOAREESUWAN, S., WILAIRATANA, P., VANNAPHAN, S., WANARATANA, V., WENISCH, C., AIKAWA, M., BRITTENHAM, G., GRANINGER, W. & WERNSDORFER, W. H. 1998. Pentoxifylline as an ancillary treatment for severe falciparum malaria in Thailand. *Am J Trop Med Hyg,* 58**,** 348-53.

LORD, C. J., MARTIN, S. A. & ASHWORTH, A. 2009. RNA interference screening demystified. *J Clin Pathol,* 62**,** 195-200.

LOWE, B. S., MOSOBO, M. & BULL, P. C. 1998. All four species of human malaria parasites form rosettes. *Trans R Soc Trop Med Hyg,* 92**,** 526.

LUCAS, J. Z. & SHERMAN, I. W. 1998. Plasmodium falciparum: thrombospondin mediates parasitized erythrocyte band 3-related adhesin binding. *Exp Parasitol,* 89**,** 78-85.
MA, F., EBIHARA, Y., UMEDA, K., SAKAI, H., HANADA, S., ZHANG, H., ZAIKE, Y., TSUCHIDA, E., NAKAHATA, T., NAKAUCHI, H. & TSUJI, K. 2008. Generation of functional erythrocytes from human embryonic stem cell-derived definitive hematopoiesis. *Proc Natl Acad Sci U S A,* 105**,** 13087-92.
MACKINNON, M. J., NDILA, C., UYOGA, S., MACHARIA, A., SNOW, R. W., BAND, G., RAUTANEN, A., ROCKETT, K. A., KWIATKOWSKI, D. P. & WILLIAMS, T. N. 2016. Environmental Correlation Analysis for Genes Associated with Protection against Malaria. *Mol Biol Evol*.
MAIER, A. G., DURAISINGH, M. T., REEDER, J. C., PATEL, S. S., KAZURA, J. W., ZIMMERMAN, P. A. & COWMAN, A. F. 2003. Plasmodium falciparum erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. *Nat Med,* 9**,** 87-92.
MAITLAND, K. 2015. Management of severe paediatric malaria in resource-limited settings. *BMC Med,* 13**,** 42.
MAITLAND, K., KIGULI, S., OPOKA, R. O., ENGORU, C., OLUPOT-OLUPOT, P., AKECH, S. O., NYEKO, R., MTOVE, G., REYBURN, H., LANG, T., BRENT, B., EVANS, J. A., TIBENDERANA, J. K., CRAWLEY, J., RUSSELL, E. C., LEVIN, M., BABIKER, A. G., GIBB, D. M. & GROUP, F. T. 2011. Mortality after fluid bolus in African children with severe infection. *N Engl J Med,* 364**,** 2483-95.
MAITLAND, K., LEVIN, M., ENGLISH, M., MITHWANI, S., PESHU, N., MARSH, K. & NEWTON, C. R. 2003. Severe P. falciparum malaria in Kenyan children: evidence for hypovolaemia. *QJM,* 96**,** 427-34.
MAITLAND, K. & NEWTON, C. R. 2005. Acidosis of severe falciparum malaria: heading for a shock? *Trends Parasitol,* 21**,** 11-6.
MAITLAND, K., PAMBA, A., ENGLISH, M., PESHU, N., LEVIN, M., MARSH, K. & NEWTON, C. R. 2005. Pre-transfusion management of children with severe malarial anaemia: a randomised controlled trial of intravascular volume expansion. *Br J Haematol,* 128**,** 393-400.
MANDAL, P. K., FERREIRA, L. M., COLLINS, R., MEISSNER, T. B., BOUTWELL, C. L., FRIESEN, M., VRBANAC, V., GARRISON, B. S., STORTCHEVOI, A., BRYDER, D., MUSUNURU, K., BRAND, H., TAGER, A. M., ALLEN, T. M., TALKOWSKI, M. E., ROSSI, D. J. & COWAN, C. A. 2014. Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. *Cell Stem Cell,* 15**,** 643-52.
MANNING, L., LAMAN, M., DAVIS, W. A. & DAVIS, T. M. 2014. Clinical features and outcome in children with severe Plasmodium falciparum malaria: a meta-analysis. *PLoS One,* 9**,** e86737.
MARCHIAFAVA, E. & BIGNAMI, A. 1894. *Two monographs on malaria and the parasites of malarial fevers. On summer-autumn malarial fevers*, New Sydenham Society.
MARSH, K., FORSTER, D., WARUIRU, C., MWANGI, I., WINSTANLEY, M., MARSH, V., NEWTON, C., WINSTANLEY, P., WARN, P. & PESHU, N. 1995. Indicators of life-threatening malaria in African children. *N Engl J Med,* 332**,** 1399-404.
MARÍN-MENÉNDEZ, A., BARDAJÍ, A., MARTÍNEZ-ESPINOSA, F. E., BÔTTO-MENEZES, C., LACERDA, M. V., ORTIZ, J., CISTERÓ, P., PIQUERAS, M., FELGER, I., MÜELLER, I., ORDI, J., DEL PORTILLO, H., MENÉNDEZ, C., WAHLGREN, M. & MAYOR, A. 2013. Rosetting in Plasmodium vivax: a cytoadhesion phenotype associated with anaemia. *PLoS Negl Trop Dis,* 7**,** e2155.
MAUDE, R. J., SILAMUT, K., PLEWES, K., CHARUNWATTHANA, P., HO, M., ABUL FAIZ, M., RAHMAN, R., HOSSAIN, M. A., HASSAN, M. U., BIN YUNUS, E., HOQUE, G., ISLAM, F., GHOSE, A., HANSON, J., SCHLATTER, J., LACEY, R., EASTAUGH, A., TARNING, J., LEE, S. J., WHITE, N. J., CHOTIVANICH, K., DAY, N. P. & DONDORP, A. M. 2014. Randomized controlled trial of levamisole hydrochloride as adjunctive therapy in severe falciparum malaria with high parasitemia. *J Infect Dis,* 209**,** 120-9.
MAYER, D. C., COFIE, J., JIANG, L., HARTL, D. L., TRACY, E., KABAT, J., MENDOZA, L. H. & MILLER, L. H. 2009. Glycophorin B is the erythrocyte receptor of Plasmodium

falciparum erythrocyte-binding ligand, EBL-1. *Proc Natl Acad Sci U S A,* 106**,** 5348-52.
MAYER, D. C., JIANG, L., ACHUR, R. N., KAKIZAKI, I., GOWDA, D. C. & MILLER, L. H. 2006. The glycophorin C N-linked glycan is a critical component of the ligand for the Plasmodium falciparum erythrocyte receptor BAEBL. *Proc Natl Acad Sci U S A,* 103**,** 2358-62.
MCGILVRAY, I. D., SERGHIDES, L., KAPUS, A., ROTSTEIN, O. D. & KAIN, K. C. 2000. Nonopsonic monocyte/macrophage phagocytosis of Plasmodium falciparum-parasitized erythrocytes: a role for CD36 in malarial clearance. *Blood,* 96**,** 3231-40.
MCMORRAN, B. J., WIECZORSKI, L., DRYSDALE, K. E., CHAN, J. A., HUANG, H. M., SMITH, C., MITIKU, C., BEESON, J. G., BURGIO, G. & FOOTE, S. J. 2012. Platelet factor 4 and Duffy antigen required for platelet killing of Plasmodium falciparum. *Science,* 338**,** 1348-51.
MERCEREAU-PUIJALON, O., GUILLOTTE, M. & VIGAN-WOMAS, I. 2008. Rosetting in Plasmodium falciparum: a cytoadherence phenotype with multiple actors. *Transfus Clin Biol,* 15**,** 62-71.
MERRYWEATHER-CLARKE, A. T., ATZBERGER, A., SONEJI, S., GRAY, N., CLARK, K., WAUGH, C., MCGOWAN, S. J., TAYLOR, S., NANDI, A. K., WOOD, W. G., ROBERTS, D. J., HIGGS, D. R., BUCKLE, V. J. & ROBSON, K. J. 2011. Global gene expression analysis of human erythroid progenitors. *Blood,* 117**,** e96-108.
MIKAMI, T. & KITAGAWA, H. 2013. Biosynthesis and function of chondroitin sulfate. *Biochim Biophys Acta,* 1830**,** 4719-33.
MILLER, L. H., BARUCH, D. I., MARSH, K. & DOUMBO, O. K. 2002. The pathogenic basis of malaria. *Nature,* 415**,** 673-9.
MILTENYI, S., MÜLLER, W., WEICHEL, W. & RADBRUCH, A. 1990. High gradient magnetic cell separation with MACS. *Cytometry,* 11**,** 231-8.
MINKAH, N. K., SCHAFER, C. & KAPPE, S. H. I. 2018. Humanized Mouse Models for the Study of Human Malaria Parasite Biology, Pathogenesis, and Immunity. *Front Immunol,* 9**,** 807.
MIZUMOTO, S., IKEGAWA, S. & SUGAHARA, K. 2013. Human genetic disorders caused by mutations in genes encoding biosynthetic enzymes for sulfated glycosaminoglycans. *J Biol Chem,* 288**,** 10953-61.
MOLES, E., MOLL, K., CH'NG, J. H., PARINI, P., WAHLGREN, M. & FERNÀNDEZ-BUSQUETS, X. 2016. Development of drug-loaded immunoliposomes for the selective targeting and elimination of rosetting Plasmodium falciparum-infected red blood cells. *J Control Release,* 241**,** 57-67.
MOLL, K., PALMKVIST, M., CH'NG, J., KIWUWA, M. S. & WAHLGREN, M. 2015. Evasion of Immunity to Plasmodium falciparum: Rosettes of Blood Group A Impair Recognition of PfEMP1. *PLoS One,* 10**,** e0145120.
MOLYNEUX, M. E., TAYLOR, T. E., WIRIMA, J. J. & BORGSTEIN, A. 1989. Clinical features and prognostic indicators in paediatric cerebral malaria: a study of 131 comatose Malawian children. *Q J Med,* 71**,** 441-59.
MOULDS, J. M. 2010. The Knops blood-group system: a review. *Immunohematology,* 26**,** 2-7.
MOULDS, J. M., MOULDS, J. J., BROWN, M. & ATKINSON, J. P. 1992. Antiglobulin testing for CR1-related (Knops/McCoy/Swain-Langley/York) blood group antigens: negative and weak reactions are caused by variable expression of CR1. *Vox Sang,* 62**,** 230-5.
MOXON, C. A., CHISALA, N. V., WASSMER, S. C., TAYLOR, T. E., SEYDEL, K. B., MOLYNEUX, M. E., FARAGHER, B., KENNEDY, N., TOH, C. H., CRAIG, A. G. & HEYDERMAN, R. S. 2014. Persistent endothelial activation and inflammation after Plasmodium falciparum Infection in Malawian children. *J Infect Dis,* 209**,** 610-5.
MOXON, C. A., HEYDERMAN, R. S. & WASSMER, S. C. 2009. Dysregulation of coagulation in cerebral malaria. *Mol Biochem Parasitol,* 166**,** 99-108.
MOXON, C. A., WASSMER, S. C., MILNER, D. A., CHISALA, N. V., TAYLOR, T. E., SEYDEL, K. B., MOLYNEUX, M. E., FARAGHER, B., ESMON, C. T., DOWNEY, C., TOH, C. H., CRAIG, A. G. & HEYDERMAN, R. S. 2013. Loss of endothelial protein C receptors links coagulation and inflammation to parasite sequestration in cerebral malaria in African children. *Blood,* 122**,** 842-51.

MU, J., AWADALLA, P., DUAN, J., MCGEE, K. M., JOY, D. A., MCVEAN, G. A. & SU, X. Z. 2005. Recombination hotspots and population structure in Plasmodium falciparum. *PLoS Biol,* 3**,** e335.
MÜNGER, K. & HOWLEY, P. M. 2002. Human papillomavirus immortalization and transformation functions. *Virus Res,* 89**,** 213-28.
NAGAYASU, E., ITO, M., AKAKI, M., NAKANO, Y., KIMURA, M., LOOAREESUWAN, S. & AIKAWA, M. 2001. CR1 density polymorphism on erythrocytes of falciparum malaria patients in Thailand. *Am J Trop Med Hyg,* 64**,** 1-5.
NANDAKUMAR, S. K., ULIRSCH, J. C. & SANKARAN, V. G. 2016. Advances in understanding erythropoiesis: evolving perspectives. *Br J Haematol,* 173**,** 206-18.
NDILA, C. M., UYOGA, S., MACHARIA, A. W., NYUTU, G., PESHU, N., OJAL, J., SHEBE, M., AWUONDO, K. O., MTURI, N., TSOFA, B., SEPÚLVEDA, N., CLARK, T. G., BAND, G., CLARKE, G., ROWLANDS, K., HUBBART, C., JEFFREYS, A., KARIUKI, S., MARSH, K., MACKINNON, M., MAITLAND, K., KWIATKOWSKI, D. P., ROCKETT, K. A., WILLIAMS, T. N. & CONSORTIUM, M. 2018. Human candidate gene polymorphisms and risk of severe malaria in children in Kilifi, Kenya: a case-control association study. *Lancet Haematol,* 5**,** e333-e345.
NETWORK, Malaria. Genome. Expression. 2014. Reappraisal of known malaria resistance loci in a large multicenter study. *Nat Genet,* 46**,** 1197-204.
NEWBOLD, C., WARN, P., BLACK, G., BERENDT, A., CRAIG, A., SNOW, B., MSOBO, M., PESHU, N. & MARSH, K. 1997. Receptor-specific adhesion and clinical disease in Plasmodium falciparum. *Am J Trop Med Hyg,* 57**,** 389-98.
NIANG, M., BEI, A. K., MADNANI, K. G., PELLY, S., DANKWA, S., KANJEE, U., GUNALAN, K., AMALADOSS, A., YEO, K. P., BOB, N. S., MALLERET, B., DURAISINGH, M. T. & PREISER, P. R. 2014. STEVOR is a Plasmodium falciparum erythrocyte binding protein that mediates merozoite invasion and rosetting. *Cell Host Microbe,* 16**,** 81-93.
NORMARK, J., NILSSON, D., RIBACKE, U., WINTER, G., MOLL, K., WHEELOCK, C. E., BAYARUGABA, J., KIRONDE, F., EGWANG, T. G., CHEN, Q., ANDERSSON, B. & WAHLGREN, M. 2007. PfEMP1-DBL1alpha amino acid motifs in severe disease states of Plasmodium falciparum malaria. *Proc Natl Acad Sci U S A,* 104**,** 15835-40.
O'DOHERTY, U., SWIGGARD, W. J. & MALIM, M. H. 2000. Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. *J Virol,* 74**,** 10074-80.
O'KEEFE, E. 2013. siRNAs and shRNAs: Tools for Protein Knockdown by Gene Silencing. *Materials and Methods* [Online]. Available: https://www.labome.com/method/siRNAs-and-shRNAs-Tools-for-Protein-Knockdown-by-Gene-Silencing.html#ref3.
O'SULLIVAN, J. M., PRESTON, R. J., O'REGAN, N. & O'DONNELL, J. S. 2016. Emerging roles for hemostatic dysfunction in malaria pathogenesis. *Blood,* 127**,** 2281-8.
OKIRO, E. A., AL-TAIAR, A., REYBURN, H., IDRO, R., BERKLEY, J. A. & SNOW, R. W. 2009. Age patterns of severe paediatric malaria and their relationship to Plasmodium falciparum transmission intensity. *Malar J,* 8**,** 4.
OKUNO, Y., SUZUKI, A., ICHIBA, S., TAKAHASHI, T., NAKAMURA, K., HITOMI, K., SASAKI, R., TADA, K. & IMURA, H. 1990. Establishment of an erythroid cell line (JK-1) that spontaneously differentiates to red cells. *Cancer,* 66**,** 1544-51.
OLIVIER, E. N., MARENAH, L., MCCAHILL, A., CONDIE, A., COWAN, S. & MOUNTFORD, J. C. 2016. High-Efficiency Serum-Free Feeder-Free Erythroid Differentiation of Human Pluripotent Stem Cells Using Small Molecules. *Stem Cells Transl Med,* 5**,** 1394-1405.
OLUPOT-OLUPOT, P., ENGORU, C., THOMPSON, J., NTEZIYAREMYE, J., CHEBET, M., SSENYONDO, T., DAMBISYA, C. M., OKUUNY, V., WOKULIRA, R., AMORUT, D., ONGODIA, P., MPOYA, A., WILLIAMS, T. N., UYOGA, S., MACHARIA, A., GIBB, D. M., WALKER, A. S. & MAITLAND, K. 2014. Phase II trial of standard versus increased transfusion volume in Ugandan children with acute severe anemia. *BMC Med,* 12**,** 67.
OPI, D. H., OCHOLA, L. B., TENDWA, M., SIDDONDO, B. R., OCHOLLA, H., FANJO, H., GHUMRA, A., FERGUSON, D. J., ROWE, J. A. & WILLIAMS, T. N. 2014. Mechanistic Studies of the Negative Epistatic Malaria-protective Interaction Between Sickle Cell Trait and α(+)thalassemia. *EBioMedicine,* 1**,** 29-36.

OPI, D. H., SWANN, O., MACHARIA, A., UYOGA, S., BAND, G., NDILA, C. M., HARRISON, E., THERA, M. A., KONE, A. K., DIALLO, D. A., DOUMBO, O. K., LYKE, K. E., PLOWE, C., MOULDS, J. M., SHEBBE, M., MTURI, N., PESHU, N., MAITLAND, K., RAZA, A., KWIATKOWSKI, D. P., ROCKETT, K. A., WILLIAMS, T. & ROWE, J. A. 2018. Two complement receptor one alleles have opposing associations with cerebral malaria and interact with α. *Elife,* 7.

OPI, D. H., UYOGA, S., ORORI, E. N., WILLIAMS, T. N. & ROWE, J. A. 2016. Red blood cell complement receptor one level varies with Knops blood group, α(+)thalassaemia and age among Kenyan children. *Genes Immun,* 17**,** 171-8.

PAIN, A., FERGUSON, D. J., KAI, O., URBAN, B. C., LOWE, B., MARSH, K. & ROBERTS, D. J. 2001. Platelet-mediated clumping of Plasmodium falciparum-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. *Proc Natl Acad Sci U S A,* 98**,** 1805-10.

PANDA, A. K., PANDA, M., TRIPATHY, R., PATTANAIK, S. S., RAVINDRAN, B. & DAS, B. K. 2012. Complement receptor 1 variants confer protection from severe malaria in Odisha, India. *PLoS One,* 7**,** e49420.

PARPIA, A. S., NDEFFO-MBAH, M. L., WENZEL, N. S. & GALVANI, A. P. 2016. Effects of Response to 2014-2015 Ebola Outbreak on Deaths from Malaria, HIV/AIDS, and Tuberculosis, West Africa. *Emerg Infect Dis,* 22**,** 433-41.

PASVOL, G., CHASIS, J. A., MOHANDAS, N., ANSTEE, D. J., TANNER, M. J. & MERRY, A. H. 1989. Inhibition of malarial parasite invasion by monoclonal antibodies against glycophorin A correlates with reduction in red cell membrane deformability. *Blood,* 74**,** 1836-43.

PATEL, S. S., MEHLOTRA, R. K., KASTENS, W., MGONE, C. S., KAZURA, J. W. & ZIMMERMAN, P. A. 2001. The association of the glycophorin C exon 3 deletion with ovalocytosis and malaria susceptibility in the Wosera, Papua New Guinea. *Blood,* 98**,** 3489-91.

PERROTTA, S., BORRIELLO, A., SCALONI, A., DE FRANCESCHI, L., BRUNATI, A. M., TURRINI, F., NIGRO, V., DEL GIUDICE, E. M., NOBILI, B., CONTE, M. L., ROSSI, F., IOLASCON, A., DONELLA-DEANA, A., ZAPPIA, V., POGGI, V., ANONG, W., LOW, P., MOHANDAS, N. & DELLA RAGIONE, F. 2005. The N-terminal 11 amino acids of human erythrocyte band 3 are critical for aldolase binding and protein phosphorylation: implications for band 3 function. *Blood,* 106**,** 4359-66.

PEYRARD, T. 2013. The LAN blood group system:a review. *Immunohematology,* 29**,** 131-5.

PIPITAPORN, B., SUEBLINVONG, T., DHARMKRONG-AT, A. & UDOMSANGPETCH, R. 2000. Rosetting of Plasmodium falciparum required multiple components of the uninfected erythrocytes. *Asian Pac J Allergy Immunol,* 18**,** 29-35.

PIVA, E., BRUGNARA, C., SPOLAORE, F. & PLEBANI, M. 2015. Clinical utility of reticulocyte parameters. *Clin Lab Med,* 35**,** 133-63.

PONGPONRATN, E., TURNER, G. D., DAY, N. P., PHU, N. H., SIMPSON, J. A., STEPNIEWSKA, K., MAI, N. T., VIRIYAVEJAKUL, P., LOOAREESUWAN, S., HIEN, T. T., FERGUSON, D. J. & WHITE, N. J. 2003. An ultrastructural study of the brain in fatal Plasmodium falciparum malaria. *Am J Trop Med Hyg,* 69**,** 345-59.

PONSFORD, M. J., MEDANA, I. M., PRAPANSILP, P., HIEN, T. T., LEE, S. J., DONDORP, A. M., ESIRI, M. M., DAY, N. P., WHITE, N. J. & TURNER, G. D. 2012. Sequestration and microvascular congestion are associated with coma in human cerebral malaria. *J Infect Dis,* 205**,** 663-71.

PRYDZ, K. & DALEN, K. T. 2000. Synthesis and sorting of proteoglycans. *J Cell Sci,* 113 Pt 2**,** 193-205.

PYZOCHA, N. K., RAN, F. A., HSU, P. D. & ZHANG, F. 2014. RNA-guided genome editing of mammalian cells. *Methods Mol Biol,* 1114**,** 269-77.

QUINTANA, M. E. P., ANGELETTI, D., MOLL, K., CHEN, Q. & WAHLGREN, M. 2016. Phagocytosis-inducing antibodies to Plasmodium falciparum upon immunization with a recombinant PfEMP1 NTS-DBL1α domain. *Malar J,* 15**,** 416.

RAHMIG, S., KRONSTEIN-WIEDEMANN, R., FOHGRUB, J., KRONSTEIN, N., NEVMERZHITSKAYA, A., BORNHÄUSER, M., GASSMANN, M., PLATZ, A., ORDEMANN, R., TONN, T. & WASKOW, C. 2016. Improved Human Erythropoiesis

and Platelet Formation in Humanized NSGW41 Mice. *Stem Cell Reports,* 7**,** 591-601.
RAMBHATLA, J. S., TURNER, L., MANNING, L., LAMAN, M., DAVIS, T. M. E., BEESON, J. G., MUELLER, I., WARREL, J., THEANDER, T. G., LAVSTSEN, T. & ROGERSON, S. J. 2019. Acquisition of Antibodies Against Endothelial Protein C Receptor-Binding Domains of Plasmodium falciparum Erythrocyte Membrane Protein 1 in Children with Severe Malaria. *J Infect Dis,* 219**,** 808-818.
RAMPENGAN, T. H. 1991. Cerebral malaria in children. Comparative study between heparin, dexamethasone and placebo. *Paediatr Indones,* 31**,** 59-66.
RAN, F. A., HSU, P. D., WRIGHT, J., AGARWALA, V., SCOTT, D. A. & ZHANG, F. 2013. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc,* 8**,** 2281-2308.
RASK, T. S., HANSEN, D. A., THEANDER, T. G., GORM PEDERSEN, A. & LAVSTSEN, T. 2010. Plasmodium falciparum erythrocyte membrane protein 1 diversity in seven genomes--divide and conquer. *PLoS Comput Biol,* 6.
RASTI, N., NAMUSOKE, F., CHÊNE, A., CHEN, Q., STAALSOE, T., KLINKERT, M. Q., MIREMBE, F., KIRONDE, F. & WAHLGREN, M. 2006. Nonimmune immunoglobulin binding and multiple adhesion characterize Plasmodium falciparum-infected erythrocytes of placental origin. *Proc Natl Acad Sci U S A,* 103**,** 13795-800.
RESENDE, M., DITLEV, S. B., NIELSEN, M. A., BODEVIN, S., BRUUN, S., PINTO, V. V., CLAUSEN, H., TURNER, L., THEANDER, T. G., SALANTI, A. & DAHLBÄCK, M. 2009. Chondroitin sulphate A (CSA)-binding of single recombinant Duffy-binding-like domains is not restricted to Plasmodium falciparum Erythrocyte Membrane Protein 1 expressed by CSA-binding parasites. *Int J Parasitol,* 39**,** 1195-204.
RIBACKE, U., MOLL, K., ALBRECHT, L., AHMED ISMAIL, H., NORMARK, J., FLABERG, E., SZEKELY, L., HULTENBY, K., PERSSON, K. E., EGWANG, T. G. & WAHLGREN, M. 2013. Improved in vitro culture of Plasmodium falciparum permits establishment of clinical isolates with preserved multiplication, invasion and rosetting phenotypes. *PLoS One,* 8**,** e69781.
RIBEIRO, M. L., ALLOISIO, N., ALMEIDA, H., GOMES, C., TEXIER, P., LEMOS, C., MIMOSO, G., MORLÉ, L., BEY-CABET, F., RUDIGOZ, R. C., DELAUNAY, J. & TAMAGNINI, G. 2000. Severe hereditary spherocytosis and distal renal tubular acidosis associated with the total absence of band 3. *Blood,* 96**,** 1602-4.
RIDGWELL, K., TANNER, M. J. & ANSTEE, D. J. 1984. The Wrb antigen in Sta-positive and Dantu-positive human erythrocytes. *J Immunogenet,* 11**,** 365-70.
RINGWALD, P., PEYRON, F., LEPERS, J. P., RABARISON, P., RAKOTOMALALA, C., RAZANAMPARANY, M., RABODONIRINA, M., ROUX, J. & LE BRAS, J. 1993. Parasite virulence factors during falciparum malaria: rosetting, cytoadherence, and modulation of cytoadherence by cytokines. *Infect Immun,* 61**,** 5198-204.
ROBERTS, D. J., CRAIG, A. G., BERENDT, A. R., PINCHES, R., NASH, G., MARSH, K. & NEWBOLD, C. I. 1992. Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature,* 357**,** 689-92.
ROBINSON, B. A., WELCH, T. L. & SMITH, J. D. 2003. Widespread functional specialization of Plasmodium falciparum erythrocyte membrane protein 1 family members to bind CD36 analysed across a parasite genome. *Mol Microbiol,* 47**,** 1265-78.
ROBSON, K., WALLIKER, D., CREASEY, A., MCBRIDE, J., BEALE, G. & WILSON, R. 1992. Cross-contamination of Plasmodium cultures. Parasitology Today.
ROGERSON, S. J., REEDER, J. C., AL-YAMAN, F. & BROWN, G. V. 1994. Sulfated glycoconjugates as disrupters of Plasmodium falciparum erythrocyte rosettes. *Am J Trop Med Hyg,* 51**,** 198-203.
ROGERSON, S. J., TEMBENU, R., DOBAÑO, C., PLITT, S., TAYLOR, T. E. & MOLYNEUX, M. E. 1999. Cytoadherence characteristics of Plasmodium falciparum-infected erythrocytes from Malawian children with severe and uncomplicated malaria. *Am J Trop Med Hyg,* 61**,** 467-72.
ROSANAS-URGELL, A., LIN, E., MANNING, L., RARAU, P., LAMAN, M., SENN, N., GRIMBERG, B. T., TAVUL, L., STANISIC, D. I., ROBINSON, L. J., APONTE, J. J., DABOD, E., REEDER, J. C., SIBA, P., ZIMMERMAN, P. A., DAVIS, T. M., KING, C. L., MICHON, P. & MUELLER, I. 2012. Reduced risk of Plasmodium vivax malaria in

Papua New Guinean children with Southeast Asian ovalocytosis in two cohorts and a case-control study. *PLoS Med,* 9**,** e1001305.
ROTTMANN, M., LAVSTSEN, T., MUGASA, J. P., KAESTLI, M., JENSEN, A. T., MÜLLER, D., THEANDER, T. & BECK, H. P. 2006. Differential expression of var gene groups is associated with morbidity caused by Plasmodium falciparum infection in Tanzanian children. *Infect Immun,* 74**,** 3904-11.
ROUT, R., DHANGADAMAJHI, G., GHADEI, M., MOHAPATRA, B. N., KAR, S. K. & RANJIT, M. 2012. Blood group phenotypes A and B are risk factors for cerebral malaria in Odisha, India. *Trans R Soc Trop Med Hyg,* 106**,** 538-43.
ROUT, R., DHANGADAMAJHI, G., MOHAPATRA, B., KAR, S. & RANJIT, M. 2010. Genetic diversity of PfEMP1-DBL 1a and it's association with severe malaria in a hyperendemic state of India. *Asian Pacific Journal of Tropical Medicine,* 3**,** 505-509.
ROUT, R., DHANGADAMAJHI, G., MOHAPATRA, B. N., KAR, S. K. & RANJIT, M. 2011. High CR1 level and related polymorphic variants are associated with cerebral malaria in eastern-India. *Infect Genet Evol,* 11**,** 139-44.
ROWE, A., BERENDT, A. R., MARSH, K. & NEWBOLD, C. I. 1994. Plasmodium falciparum: a family of sulphated glycoconjugates disrupts erythrocyte rosettes. *Exp Parasitol,* 79**,** 506-16.
ROWE, A., OBEIRO, J., NEWBOLD, C. I. & MARSH, K. 1995. Plasmodium falciparum rosetting is associated with malaria severity in Kenya. *Infect Immun,* 63**,** 2323-6.
ROWE, J. A., CLAESSENS, A., CORRIGAN, R. A. & ARMAN, M. 2009. Adhesion of Plasmodium falciparum-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications. *Expert Rev Mol Med,* 11**,** e16.
ROWE, J. A., HANDEL, I. G., THERA, M. A., DEANS, A. M., LYKE, K. E., KONÉ, A., DIALLO, D. A., RAZA, A., KAI, O., MARSH, K., PLOWE, C. V., DOUMBO, O. K. & MOULDS, J. M. 2007. Blood group O protects against severe Plasmodium falciparum malaria through the mechanism of reduced rosetting. *Proc Natl Acad Sci U S A,* 104**,** 17471-6.
ROWE, J. A., MOULDS, J. M., NEWBOLD, C. I. & MILLER, L. H. 1997. P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature,* 388**,** 292-5.
ROWE, J. A., OBIERO, J., MARSH, K. & RAZA, A. 2002a. Short report: Positive correlation between rosetting and parasitemia in Plasmodium falciparum clinical isolates. *Am J Trop Med Hyg,* 66**,** 458-60.
ROWE, J. A., RAZA, A., DIALLO, D. A., BABY, M., POUDIOUGO, B., COULIBALY, D., COCKBURN, I. A., MIDDLETON, J., LYKE, K. E., PLOWE, C. V., DOUMBO, O. K. & MOULDS, J. M. 2002b. Erythrocyte CR1 expression level does not correlate with a HindIII restriction fragment length polymorphism in Africans; implications for studies on malaria susceptibility. *Genes Immun,* 3**,** 497-500.
ROWE, J. A., ROGERSON, S. J., RAZA, A., MOULDS, J. M., KAZATCHKINE, M. D., MARSH, K., NEWBOLD, C. I., ATKINSON, J. P. & MILLER, L. H. 2000. Mapping of the region of complement receptor (CR) 1 required for Plasmodium falciparum rosetting and demonstration of the importance of CR1 in rosetting in field isolates. *J Immunol,* 165**,** 6341-6.
ROWE, J. A., SHAFI, J., KAI, O. K., MARSH, K. & RAZA, A. 2002c. Nonimmune IgM, but not IgG binds to the surface of Plasmodium falciparum-infected erythrocytes and correlates with rosetting and severe malaria. *Am J Trop Med Hyg,* 66**,** 692-9.
RUSSELL, C., MERCEREAU-PUIJALON, O., LE SCANF, C., STEWARD, M. & ARNOT, D. E. 2005. Further definition of PfEMP-1 DBL-1alpha domains mediating rosetting adhesion of Plasmodium falciparum. *Mol Biochem Parasitol,* 144**,** 109-13.
SAIWAEW, S., SRITABAL, J., PIARAKSA, N., KEAYARSA, S., RUENGWEERAYUT, R., UTAISIN, C., SILA, P., NIRAMIS, R., UDOMSANGPETCH, R., CHARUNWATTHANA, P., PONGPONRATN, E., PUKRITTAYAKAMEE, S., LEITGEB, A. M., WAHLGREN, M., LEE, S. J., DAY, N. P., WHITE, N. J., DONDORP, A. M. & CHOTIVANICH, K. 2017. Effects of sevuparin on rosette formation and cytoadherence of Plasmodium falciparum infected erythrocytes. *PLoS One,* 12**,** e0172718.

SALANTI, A., STAALSOE, T., LAVSTSEN, T., JENSEN, A. T., SOWA, M. P., ARNOT, D. E., HVIID, L. & THEANDER, T. G. 2003. Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering Plasmodium falciparum involved in pregnancy-associated malaria. *Mol Microbiol,* 49**,** 179-91.
SANJANA, N. E., SHALEM, O. & ZHANG, F. 2014. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods,* 11**,** 783-784.
SARRAZIN, S., LAMANNA, W. C. & ESKO, J. D. 2011. Heparan sulfate proteoglycans. *Cold Spring Harb Perspect Biol,* 3.
SCHERF, A., HERNANDEZ-RIVAS, R., BUFFET, P., BOTTIUS, E., BENATAR, C., POUVELLE, B., GYSIN, J. & LANZER, M. 1998. Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. *EMBO J,* 17**,** 5418-26.
SCHMIDT, C. Q., KENNEDY, A. T. & THAM, W. H. 2015. More than just immune evasion: Hijacking complement by Plasmodium falciparum. *Mol Immunol,* 67**,** 71-84.
SCHOFIELD, A. E., REARDON, D. M. & TANNER, M. J. 1992. Defective anion transport activity of the abnormal band 3 in hereditary ovalocytic red blood cells. *Nature,* 355**,** 836-8.
SCHOLANDER, C., TREUTIGER, C. J., HULTENBY, K. & WAHLGREN, M. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nat Med,* 2**,** 204-8.
SEMBLAT, J. P., GHUMRA, A., CZAJKOWSKY, D. M., WALLIS, R., MITCHELL, D. A., RAZA, A. & ROWE, J. A. 2015. Identification of the minimal binding region of a Plasmodium falciparum IgM binding PfEMP1 domain. *Mol Biochem Parasitol,* 201**,** 76-82.
SEMBLAT, J. P., RAZA, A., KYES, S. A. & ROWE, J. A. 2006. Identification of Plasmodium falciparum var1CSA and var2CSA domains that bind IgM natural antibodies. *Mol Biochem Parasitol,* 146**,** 192-7.
SHALEM, O., SANJANA, N. E., HARTENIAN, E., SHI, X., SCOTT, D. A., MIKKELSON, T., HECKL, D., EBERT, B. L., ROOT, D. E., DOENCH, J. G. & ZHANG, F. 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science,* 343**,** 84-87.
SHERMAN, I. W., CRANDALL, I. E., GUTHRIE, N. & LAND, K. M. 1995. The sticky secrets of sequestration. *Parasitol Today,* 11**,** 378-84.
SHERMAN, I. W., EDA, S. & WINOGRAD, E. 2003. Cytoadherence and sequestration in Plasmodium falciparum: defining the ties that bind. *Microbes Infect,* 5**,** 897-909.
SILAMUT, K., PHU, N. H., WHITTY, C., TURNER, G. D., LOUWRIER, K., MAI, N. T., SIMPSON, J. A., HIEN, T. T. & WHITE, N. J. 1999. A quantitative analysis of the microvascular sequestration of malaria parasites in the human brain. *Am J Pathol,* 155**,** 395-410.
SIM, B. K., CHITNIS, C. E., WASNIOWSKA, K., HADLEY, T. J. & MILLER, L. H. 1994. Receptor and ligand domains for invasion of erythrocytes by Plasmodium falciparum. *Science,* 264**,** 1941-4.
SINGH, B., KIM SUNG, L., MATUSOP, A., RADHAKRISHNAN, A., SHAMSUL, S. S., COX-SINGH, J., THOMAS, A. & CONWAY, D. J. 2004. A large focus of naturally acquired Plasmodium knowlesi infections in human beings. *Lancet,* 363**,** 1017-24.
SINGH, H., MADNANI, K., LIM, Y. B., CAO, J., PREISER, P. R. & LIM, C. T. 2016. Expression dynamics and physiologically relevant functional study of STEVOR in asexual stages of Plasmodium falciparum infection. *Cell Microbiol*.
SINHA, S., JHA, G. N., ANAND, P., QIDWAI, T., PATI, S. S., MOHANTY, S., MISHRA, S. K., TYAGI, P. K., SHARMA, S. K., VENKATESH, V. & HABIB, S. 2009. CR1 levels and gene polymorphisms exhibit differential association with falciparum malaria in regions of varying disease endemicity. *Hum Immunol,* 70**,** 244-50.
SKIDMORE, M. A., DUMAX-VORZET, A. F., GUIMOND, S. E., RUDD, T. R., EDWARDS, E. A., TURNBULL, J. E., CRAIG, A. G. & YATES, E. A. 2008. Disruption of rosetting in Plasmodium falciparum malaria with chemically modified heparin and low molecular weight derivatives possessing reduced anticoagulant and other serine protease inhibition activities. *J Med Chem,* 51**,** 1453-8.

SMITH, J. D., CHITNIS, C. E., CRAIG, A. G., ROBERTS, D. J., HUDSON-TAYLOR, D. E., PETERSON, D. S., PINCHES, R., NEWBOLD, C. I. & MILLER, L. H. 1995. Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell,* 82**,** 101-10.

SMITH, J. D., ROWE, J. A., HIGGINS, M. K. & LAVSTSEN, T. 2013. Malaria's deadly grip: cytoadhesion of Plasmodium falciparum-infected erythrocytes. *Cell Microbiol,* 15**,** 1976-83.

SMITSKAMP, H. & WOLTHUIS, F. H. 1971. New concepts in treatment of malignant tertian malaria with cerebral involvement. *Br Med J,* 1**,** 714-6.

SNOW, R. W., BASTOS DE AZEVEDO, I., LOWE, B. S., KABIRU, E. W., NEVILL, C. G., MWANKUSYE, S., KASSIGA, G., MARSH, K. & TEUSCHER, T. 1994. Severe childhood malaria in two areas of markedly different falciparum transmission in east Africa. *Acta Trop,* 57**,** 289-300.

SNOW, R. W., OMUMBO, J. A., LOWE, B., MOLYNEUX, C. S., OBIERO, J. O., PALMER, A., WEBER, M. W., PINDER, M., NAHLEN, B., OBONYO, C., NEWBOLD, C., GUPTA, S. & MARSH, K. 1997. Relation between severe malaria morbidity in children and level of Plasmodium falciparum transmission in Africa. *Lancet,* 349**,** 1650-4.

SQUIRES, A., NASEF, N., LIN, Y., CALLUM, J., KHADAWARDI, E. M., DROLET, C., CORE, D. & SIMMONS, B. 2012. Hemolytic disease of the newborn caused by anti-Wright (anti-Wra): case report and review of literature. *Neonatal Netw,* 31**,** 69-80.

SRIKANTH, C. B., SALIMATH, P. V. & NANDINI, C. D. 2012. Erythrocytes express chondroitin sulphate/dermatan sulphate, which undergoes quantitative changes during diabetes and mediate erythrocyte adhesion to extracellular matrix components. *Biochimie,* 94**,** 1347-55.

STAALSOE, T., GIHA, H. A., DODOO, D., THEANDER, T. G. & HVIID, L. 1999. Detection of antibodies to variant antigens on Plasmodium falciparum-infected erythrocytes by flow cytometry. *Cytometry,* 35**,** 329-36.

STELZER, G., ROSEN, N., PLASCHKES, I., ZIMMERMAN, S., TWIK, M., FISHILEVICH, S., STEIN, T. I., NUDEL, R., LIEDER, I., MAZOR, Y., KAPLAN, S., DAHARY, D., WARSHAWSKY, D., GUAN-GOLAN, Y., KOHN, A., RAPPAPORT, N., SAFRAN, M. & LANCET, D. 2016. The GeneCards Suite: From Gene Data Mining to Disease Genome Sequence Analyses. *Curr Protoc Bioinformatics,* 54**,** 1.30.1-1.30.33.

STEVENSON, L., HUDA, P., JEPPESEN, A., LAURSEN, E., ROWE, J. A., CRAIG, A., STREICHER, W., BARFOD, L. & HVIID, L. 2015a. Investigating the function of Fc-specific binding of IgM to Plasmodium falciparum erythrocyte membrane protein 1 mediating erythrocyte rosetting. *Cell Microbiol,* 17**,** 819-31.

STEVENSON, L., LAURSEN, E., COWAN, G. J., BANDOH, B., BARFOD, L., CAVANAGH, D. R., ANDERSEN, G. R. & HVIID, L. 2015b. α2-Macroglobulin Can Crosslink Multiple Plasmodium falciparum Erythrocyte Membrane Protein 1 (PfEMP1) Molecules and May Facilitate Adhesion of Parasitized Erythrocytes. *PLoS Pathog,* 11**,** e1005022.

STORM, J., JESPERSEN, J. S., SEYDEL, K. B., SZESTAK, T., MBEWE, M., CHISALA, N. V., PHULA, P., WANG, C. W., TAYLOR, T. E., MOXON, C. A., LAVSTSEN, T. & CRAIG, A. G. 2019. Cerebral malaria is associated with differential cytoadherence to brain endothelial cells. *EMBO Mol Med,* 11.

STOUTE, J. A. 2011. Complement receptor 1 and malaria. *Cell Microbiol,* 13**,** 1441-50.

SU, X. Z., HEATWOLE, V. M., WERTHEIMER, S. P., GUINET, F., HERRFELDT, J. A., PETERSON, D. S., RAVETCH, J. A. & WELLEMS, T. E. 1995. The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. *Cell,* 82**,** 89-100.

SUBRAMANI, R., QUADT, K., JEPPESEN, A. E., HEMPEL, C., PETERSEN, J. E., HASSENKAM, T., HVIID, L. & BARFOD, L. 2015. Plasmodium falciparum-infected erythrocyte knob density is linked to the PfEMP1 variant expressed. *MBio,* 6**,** e01456-15.

TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K. & YAMANAKA, S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell,* 131**,** 861-72.

TAKAHASHI, K. & YAMANAKA, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell,* 126**,** 663-76.
TAMHANKAR, M., GERHARDT, D. M., BENNETT, R. S., MURPHY, N., JAHRLING, P. B. & PATTERSON, J. L. 2018. Heparan sulfate is an important mediator of Ebola virus infection in polarized epithelial cells. *Virol J,* 15**,** 135.
TAYLOR, T. E., FU, W. J., CARR, R. A., WHITTEN, R. O., MUELLER, J. S., FOSIKO, N. G., LEWALLEN, S., LIOMBA, N. G., MOLYNEUX, M. E. & MUELLER, J. G. 2004. Differentiating the pathologies of cerebral malaria by postmortem parasite counts. *Nat Med,* 10**,** 143-5.
TAYLOR, T. E., MOLYNEUX, M. E., WIRIMA, J. J., BORGSTEIN, A., GOLDRING, J. D. & HOMMEL, M. 1992. Intravenous immunoglobulin in the treatment of paediatric cerebral malaria. *Clin Exp Immunol,* 90**,** 357-62.
TEERANAIPONG, P., OHASHI, J., PATARAPOTIKUL, J., KIMURA, R., NUCHNOI, P., HANANANTACHAI, H., NAKA, I., PUTAPORNTIP, C., JONGWUTIWES, S. & TOKUNAGA, K. 2008. A functional single-nucleotide polymorphism in the CR1 promoter region contributes to protection against cerebral malaria. *J Infect Dis,* 198**,** 1880-91.
TEKESTE, Z. & PETROS, B. 2010. The ABO blood group and Plasmodium falciparum malaria in Awash, Metehara and Ziway areas, Ethiopia. *Malar J,* 9**,** 280.
TELEN, M. J., LE VAN KIM, C., CHUNG, A., CARTRON, J. P. & COLIN, Y. 1991. Molecular basis for elliptocytosis associated with glycophorin C and D deficiency in the Leach phenotype. *Blood,* 78**,** 1603-6.
TEMBO, D. L., NYONI, B., MURIKOLI, R. V., MUKAKA, M., MILNER, D. A., BERRIMAN, M., ROGERSON, S. J., TAYLOR, T. E., MOLYNEUX, M. E., MANDALA, W. L., CRAIG, A. G. & MONTGOMERY, J. 2014. Differential PfEMP1 expression is associated with cerebral malaria pathology. *PLoS Pathog,* 10**,** e1004537.
TETTEH-QUARCOO, P. B., SCHMIDT, C. Q., THAM, W. H., HAUHART, R., MERTENS, H. D., ROWE, A., ATKINSON, J. P., COWMAN, A. F., ROWE, J. A. & BARLOW, P. N. 2012. Lack of evidence from studies of soluble protein fragments that Knops blood group polymorphisms in complement receptor-type 1 are driven by malaria. *PLoS One,* 7**,** e34820.
THAM, W. H., WILSON, D. W., LOPATICKI, S., SCHMIDT, C. Q., TETTEH-QUARCOO, P. B., BARLOW, P. N., RICHARD, D., CORBIN, J. E., BEESON, J. G. & COWMAN, A. F. 2010. Complement receptor 1 is the host erythrocyte receptor for Plasmodium falciparum PfRh4 invasion ligand. *Proc Natl Acad Sci U S A,* 107**,** 17327-32.
THERON, M., CROSS, N., CAWKILL, P., BUSTAMANTE, L. Y. & RAYNER, J. C. 2018. An in vitro erythrocyte preference assay reveals that Plasmodium falciparum parasites prefer Type O over Type A erythrocytes. *Sci Rep,* 8**,** 8133.
TOYE, A. M., WILLIAMSON, R. C., KHANFAR, M., BADER-MEUNIER, B., CYNOBER, T., THIBAULT, M., TCHERNIA, G., DÉCHAUX, M., DELAUNAY, J. & BRUCE, L. J. 2008. Band 3 Courcouronnes (Ser667Phe): a trafficking mutant differentially rescued by wild-type band 3 and glycophorin A. *Blood,* 111**,** 5380-9.
TRAKARNSANGA, K., GRIFFITHS, R. E., WILSON, M. C., BLAIR, A., SATCHWELL, T. J., MEINDERS, M., COGAN, N., KUPZIG, S., KURITA, R., NAKAMURA, Y., TOYE, A. M., ANSTEE, D. J. & FRAYNE, J. 2017. An immortalized adult human erythroid line facilitates sustainable and scalable generation of functional red cells. *Nat Commun,* 8**,** 14750.
TREEPRASERTSUK, S., KRUDSOOD, S., TOSUKHOWONG, T., MAEK-A-NANTAWAT, W., VANNAPHAN, S., SAENGNETSWANG, T., LOOAREESUWAN, S., KUHN, W. F., BRITTENHAM, G. & CARROLL, J. 2003. N-acetylcysteine in severe falciparum malaria in Thailand. *Southeast Asian J Trop Med Public Health,* 34**,** 37-42.
TREUTIGER, C. J., HEDLUND, I., HELMBY, H., CARLSON, J., JEPSON, A., TWUMASI, P., KWIATKOWSKI, D., GREENWOOD, B. M. & WAHLGREN, M. 1992. Rosette formation in Plasmodium falciparum isolates and anti-rosette activity of sera from Gambians with cerebral or uncomplicated malaria. *Am J Trop Med Hyg,* 46**,** 503-10.
TURNER, L., LAVSTSEN, T., BERGER, S. S., WANG, C. W., PETERSEN, J. E., AVRIL, M., BRAZIER, A. J., FREETH, J., JESPERSEN, J. S., NIELSEN, M. A., MAGISTRADO, P., LUSINGU, J., SMITH, J. D., HIGGINS, M. K. & THEANDER, T. G. 2013. Severe

malaria is associated with parasite binding to endothelial protein C receptor. *Nature,* 498**,** 502-5.
UDOMSANGPETCH, R., TODD, J., CARLSON, J. & GREENWOOD, B. M. 1993. The effects of hemoglobin genotype and ABO blood group on the formation of rosettes by Plasmodium falciparum-infected red blood cells. *Am J Trop Med Hyg,* 48**,** 149-53.
UDOMSANGPETCH, R., WÅHLIN, B., CARLSON, J., BERZINS, K., TORII, M., AIKAWA, M., PERLMANN, P. & WAHLGREN, M. 1989. Plasmodium falciparum-infected erythrocytes form spontaneous erythrocyte rosettes. *J Exp Med,* 169**,** 1835-40.
UDOMSANPETCH, R., THANIKKUL, K., PUKRITTAYAKAMEE, S. & WHITE, N. J. 1995. Rosette formation by Plasmodium vivax. *Trans R Soc Trop Med Hyg,* 89**,** 635-7.
VAN HENSBROEK, M. B., PALMER, A., ONYIORAH, E., SCHNEIDER, G., JAFFAR, S., DOLAN, G., MEMMING, H., FRENKEL, J., ENWERE, G., BENNETT, S., KWIATKOWSKI, D. & GREENWOOD, B. 1996. The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria. *J Infect Dis,* 174**,** 1091-7.
VARKI , A., CUMMINGS , R., ESKO , J., FREEZE , H., STANLEY , P., BERTOZZI , C., HART , G. & ETZLER, M. 2009. *Essentials of Glycobiology,* New York, Cold Spring Harbor.
VENGELEN-TYLER, V., ANSTEE, D. J., ISSITT, P. D., PAVONE, B. G., FERGUSON, S. J., MAWBY, W. J., TANNER, M. J., BLAJCHMAN, M. A. & LORQUE, P. 1981. Studies on the blood of an MiV homozygote. *Transfusion,* 21**,** 1-14.
VIGAN-WOMAS, I., GUILLOTTE, M., JUILLERAT, A., HESSEL, A., RAYNAL, B., ENGLAND, P., COHEN, J. H., BERTRAND, O., PEYRARD, T., BENTLEY, G. A., LEWIT-BENTLEY, A. & MERCEREAU-PUIJALON, O. 2012. Structural basis for the ABO blood-group dependence of Plasmodium falciparum rosetting. *PLoS Pathog,* 8**,** e1002781.
VIGAN-WOMAS, I., GUILLOTTE, M., JUILLERAT, A., VALLIERES, C., LEWIT-BENTLEY, A., TALL, A., BARIL, L., BENTLEY, G. A. & MERCEREAU-PUIJALON, O. 2011. Allelic diversity of the Plasmodium falciparum erythrocyte membrane protein 1 entails variant-specific red cell surface epitopes. *PLoS One,* 6**,** e16544.
VIGAN-WOMAS, I., GUILLOTTE, M., LE SCANF, C., IGONET, S., PETRES, S., JUILLERAT, A., BADAUT, C., NATO, F., SCHNEIDER, A., LAVERGNE, A., CONTAMIN, H., TALL, A., BARIL, L., BENTLEY, G. A. & MERCEREAU-PUIJALON, O. 2008. An in vivo and in vitro model of Plasmodium falciparum rosetting and autoagglutination mediated by varO, a group A var gene encoding a frequent serotype. *Infect Immun,* 76**,** 5565-80.
VIGAN-WOMAS, I., LOKOSSOU, A., GUILLOTTE, M., JUILLERAT, A., BENTLEY, G., GARCIA, A., MERCEREAU-PUIJALON, O. & MIGOT-NABIAS, F. 2010. The humoral response to Plasmodium falciparum VarO rosetting variant and its association with protection against malaria in Beninese children. *Malar J,* 9**,** 267.
VINJAMUR, D. S. & BAUER, D. E. 2018. Growing and Genetically Manipulating Human Umbilical Cord Blood-Derived Erythroid Progenitor (HUDEP) Cell Lines. *Methods Mol Biol,* 1698**,** 275-284.
VOGT, A. M., BARRAGAN, A., CHEN, Q., KIRONDE, F., SPILLMANN, D. & WAHLGREN, M. 2003. Heparan sulfate on endothelial cells mediates the binding of Plasmodium falciparum-infected erythrocytes via the DBL1alpha domain of PfEMP1. *Blood,* 101**,** 2405-11.
VOGT, A. M., PETTERSSON, F., MOLL, K., JONSSON, C., NORMARK, J., RIBACKE, U., EGWANG, T. G., EKRE, H. P., SPILLMANN, D., CHEN, Q. & WAHLGREN, M. 2006. Release of sequestered malaria parasites upon injection of a glycosaminoglycan. *PLoS Pathog,* 2**,** e100.
VOGT, A. M., WINTER, G., WAHLGREN, M. & SPILLMANN, D. 2004. Heparan sulphate identified on human erythrocytes: a Plasmodium falciparum receptor. *Biochem J,* 381**,** 593-7.
VON SEIDLEIN, L., OLAOSEBIKAN, R., HENDRIKSEN, I. C., LEE, S. J., ADEDOYIN, O. T., AGBENYEGA, T., NGUAH, S. B., BOJANG, K., DEEN, J. L., EVANS, J., FANELLO, C. I., GOMES, E., PEDRO, A. J., KAHABUKA, C., KAREMA, C., KIVAYA, E., MAITLAND, K., MOKUOLU, O. A., MTOVE, G., MWANGA-AMUMPAIRE, J.,

NADJM, B., NANSUMBA, M., NGUM, W. P., ONYAMBOKO, M. A., REYBURN, H., SAKULTHAEW, T., SILAMUT, K., TSHEFU, A. K., UMULISA, N., GESASE, S., DAY, N. P., WHITE, N. J. & DONDORP, A. M. 2012. Predicting the clinical outcome of severe falciparum malaria in african children: findings from a large randomized trial. *Clin Infect Dis,* 54**,** 1080-90.

WAHLGREN, M., ABRAMS, J. S., FERNANDEZ, V., BEJARANO, M. T., AZUMA, M., TORII, M., AIKAWA, M. & HOWARD, R. J. 1995. Adhesion of Plasmodium falciparum-infected erythrocytes to human cells and secretion of cytokines (IL-1-beta, IL-1RA, IL-6, lL-8, IL-10, TGF beta, TNF alpha, G-CSF, GM-CSF. *Scand J Immunol,* 42**,** 626-36.

WAHLGREN, M., CARLSON, J., HELMBY, H., HEDLUND, I. & TREUTIGER, C. J. 1992. Molecular mechanisms and biological importance of Plasmodium falciparum erythrocyte rosetting. *Mem Inst Oswaldo Cruz,* 87 Suppl 3**,** 323-9.

WAHLGREN, M., CARLSON, J., UDOMSANGPETCH, R. & PERLMANN, P. 1989. Why do Plasmodium falciparumm-infected erythrocytes form spontaneous erythrocyte rosettes? *Parasitol Today,* 5**,** 183-5.

WALLER, K. L., COOKE, B. M., NUNOMURA, W., MOHANDAS, N. & COPPEL, R. L. 1999. Mapping the binding domains involved in the interaction between the Plasmodium falciparum knob-associated histidine-rich protein (KAHRP) and the cytoadherence ligand P. falciparum erythrocyte membrane protein 1 (PfEMP1). *J Biol Chem,* 274**,** 23808-13.

WARIMWE, G. M., FEGAN, G., MUSYOKI, J. N., NEWTON, C. R., OPIYO, M., GITHINJI, G., ANDISI, C., MENZA, F., KITSAO, B., MARSH, K. & BULL, P. C. 2012. Prognostic indicators of life-threatening malaria are associated with distinct parasite variant antigen profiles. *Sci Transl Med,* 4**,** 129ra45.

WARIMWE, G. M., KEANE, T. M., FEGAN, G., MUSYOKI, J. N., NEWTON, C. R., PAIN, A., BERRIMAN, M., MARSH, K. & BULL, P. C. 2009. Plasmodium falciparum var gene expression is modified by host immunity. *Proc Natl Acad Sci U S A,* 106**,** 21801-6.

WARRELL, D. A., LOOAREESUWAN, S., WARRELL, M. J., KASEMSARN, P., INTARAPRASERT, R., BUNNAG, D. & HARINASUTA, T. 1982. Dexamethasone proves deleterious in cerebral malaria. A double-blind trial in 100 comatose patients. *N Engl J Med,* 306**,** 313-9.

WASSMER, S. C. & GRAU, G. E. 2017. Severe malaria: what's new on the pathogenesis front? *Int J Parasitol,* 47**,** 145-152.

WASSMER, S. C., MOXON, C. A., TAYLOR, T., GRAU, G. E., MOLYNEUX, M. E. & CRAIG, A. G. 2011. Vascular endothelial cells cultured from patients with cerebral or uncomplicated malaria exhibit differential reactivity to TNF. *Cell Microbiol,* 13**,** 198-209.

WASSMER, S. C., TAYLOR, T. E., RATHOD, P. K., MISHRA, S. K., MOHANTY, S., AREVALO-HERRERA, M., DURAISINGH, M. T. & SMITH, J. D. 2015. Investigating the Pathogenesis of Severe Malaria: A Multidisciplinary and Cross-Geographical Approach. *Am J Trop Med Hyg,* 93**,** 42-56.

WATT, G., JONGSAKUL, K. & RUANGVIRAYUTH, R. 2002. A pilot study of N-acetylcysteine as adjunctive therapy for severe malaria. *QJM,* 95**,** 285-90.

WHITE, N. J., LOOAREESUWAN, S., PHILLIPS, R. E., CHANTHAVANICH, P. & WARRELL, D. A. 1988. Single dose phenobarbitone prevents convulsions in cerebral malaria. *Lancet,* 2**,** 64-6.

WHITE, N. J., PUKRITTAYAKAMEE, S., HIEN, T. T., FAIZ, M. A., MOKUOLU, O. A. & DONDORP, A. M. 2014. Malaria. *Lancet,* 383**,** 723-35.

WHITE, N. J., TURNER, G. D., DAY, N. P. & DONDORP, A. M. 2013. Lethal malaria: Marchiafava and Bignami were right. *J Infect Dis,* 208**,** 192-8.

WHO 1986. Severe and complicated malaria. World Health Organization Malaria Action Programme. *Trans R Soc Trop Med Hyg,* 80 Suppl**,** 3-50.

WHO 2012. *Management of Severe Malaria: A practical handbook*, World Health Organization.

WHO 2014. Severe malaria. *Trop Med Int Health,* 19 Suppl 1**,** 7-131.

WHO 2015a. *Guidelines for the treatment of malaria*, World Health organization.

WHO 2015b. Health worker Ebola infections in Guinea, Liberia and Sierra LeonePreliminary report. Geneva.
WHO 2017. World malaria report 2017.
WHO 2018. World Health Organization Malaria Report 2018. Geneva.
WIEDENHEFT, B., STERNBERG, S. H. & DOUDNA, J. A. 2012. RNA-guided genetic silencing systems in bacteria and archaea. *Nature,* 482**,** 331-8.
WILSON, M. C., TRAKARNSANGA, K., HEESOM, K. J., COGAN, N., GREEN, C., TOYE, A. M., PARSONS, S. F., ANSTEE, D. J. & FRAYNE, J. 2016. Comparison of the Proteome of Adult and Cord Erythroid Cells, and Changes in the Proteome Following Reticulocyte Maturation. *Mol Cell Proteomics,* 15**,** 1938-46.
WINOGRAD, E., EDA, S. & SHERMAN, I. W. 2004. Chemical modifications of band 3 protein affect the adhesion of Plasmodium falciparum-infected erythrocytes to CD36. *Mol Biochem Parasitol,* 136**,** 243-8.
WINOGRAD, E., PRUDHOMME, J. G. & SHERMAN, I. W. 2005. Band 3 clustering promotes the exposure of neoantigens in Plasmodium falciparum-infected erythrocytes. *Mol Biochem Parasitol,* 142**,** 98-105.
WINOGRAD, E. & SHERMAN, I. W. 1989. Characterization of a modified red cell membrane protein expressed on erythrocytes infected with the human malaria parasite Plasmodium falciparum: possible role as a cytoadherent mediating protein. *J Cell Biol,* 108**,** 23-30.
WINOGRAD, E. & SHERMAN, I. W. 2004. Malaria infection induces a conformational change in erythrocyte band 3 protein. *Mol Biochem Parasitol,* 138**,** 83-7.
WOOD, B. 2004. Multicolor immunophenotyping: human immune system hematopoiesis. *Methods Cell Biol,* 75**,** 559-76.
YAM, X. Y., NIANG, M., MADNANI, K. G. & PREISER, P. R. 2017. Three Is a Crowd - New Insights into Rosetting in Plasmodium falciparum. *Trends Parasitol,* 33**,** 309-320.
YAN, R., ZHANG, Y., CAI, D., LIU, Y., CUCONATI, A. & GUO, H. 2015. Spinoculation Enhances HBV Infection in NTCP-Reconstituted Hepatocytes. *PLoS One,* 10**,** e0129889.
YANG, C. T., MA, R., AXTON, R. A., JACKSON, M., TAYLOR, A. H., FIDANZA, A., MARENAH, L., FRAYNE, J., MOUNTFORD, J. C. & FORRESTER, L. M. 2017. Activation of KLF1 Enhances the Differentiation and Maturation of Red Blood Cells from Human Pluripotent Stem Cells. *Stem Cells,* 35**,** 886-897.
YEO, T. W., LAMPAH, D. A., ROOSLAMIATI, I., GITAWATI, R., TJITRA, E., KENANGALEM, E., PRICE, R. N., DUFFULL, S. B. & ANSTEY, N. M. 2013. A randomized pilot study of L-arginine infusion in severe falciparum malaria: preliminary safety, efficacy and pharmacokinetics. *PLoS One,* 8**,** e69587.
YOUNG, M. T., BECKMANN, R., TOYE, A. M. & TANNER, M. J. 2000. Red-cell glycophorin A-band 3 interactions associated with the movement of band 3 to the cell surface. *Biochem J,* 350 Pt 1**,** 53-60.
YU, K. R., NATANSON, H. & DUNBAR, C. E. 2016. Gene Editing of Human Hematopoietic Stem and Progenitor Cells: Promise and Potential Hurdles. *Hum Gene Ther,* 27**,** 729-740.
YU, L. C., TWU, Y. C., CHANG, C. Y. & LIN, M. 2001. Molecular basis of the adult i phenotype and the gene responsible for the expression of the human blood group I antigen. *Blood,* 98**,** 3840-5.
ZEUNER, A., MARTELLI, F., VAGLIO, S., FEDERICI, G., WHITSETT, C. & MIGLIACCIO, A. R. 2012. Concise review: stem cell-derived erythrocytes as upcoming players in blood transfusion. *Stem Cells,* 30**,** 1587-96.
ZHANG, Y., NESTER, C. M., HOLANDA, D. G., MARSH, H. C., HAMMOND, R. A., THOMAS, L. J., MEYER, N. C., HUNSICKER, L. G., SETHI, S. & SMITH, R. J. 2013. Soluble CR1 therapy improves complement regulation in C3 glomerulopathy. *J Am Soc Nephrol,* 24**,** 1820-9.